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<p>(21) International Application Number: PCT/US91/02209 (22) International Filing Date: 4 April 1991 (04.04.91) (30) Priority data: 505,434 4 April 1990 (04.04.90) US (71) Applicant: PROTOS, INC. [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US). (72) Inventor: ROSENBERG, Steven ; 2323 Bywood Drive, Oakland, CA 94602 (US). (74) Agents: CIOTTI, Thomas, E. et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025-3471 (US).</p>		<p>(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (Euro- pean patent), FI, FR (European patent), GA (OAPI pa- tent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (Euro- pean patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OA- PI patent), TG (OAPI patent).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: HEPATITIS C VIRUS PROTEASE INHIBITORS</p> <p>(57) Abstract</p> <p>A method for assaying compounds for activity against Hepatitis C virus by contacting an inactive HCV analog with a mixture of candidate anti-HCV compounds and determining which candidate compounds bind to said HCV protease analog.</p>		

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- 1 -

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HEPATITIS C VIRUS PROTEASE INHIBITORSCross-Reference to Related Application

10 This application is a continuation-in-part application of U.S. Serial No. 07/505,434, filed 4 April 1990.

Technical Field

15 This invention relates to the molecular biology and virology of the hepatitis C virus (HCV). More specifically, this invention relates to a novel protease produced by HCV, methods of expression, recombinant protease, protease mutants, and inhibitors of HCV protease.

Background of the Invention

20 Non-A, Non-B hepatitis (NANBH) is a transmissible disease (or family of diseases) that is believed to be virally induced, and is distinguishable from other forms of virus-associated liver disease, such as those caused by hepatitis A virus (HAV), hepatitis B virus (HBV), delta hepatitis virus (HDV), cytomegalovirus (CMV) or Epstein-Barr virus (EBV). Epidemiologic evidence suggests that there may be three
25 types of NANBH: the water-borne epidemic type; the blood or needle associated type; and the sporadically occurring (community acquired) type. However, the number of causative agents is unknown. Recently, however, a new viral species, hepatitis C virus (HCV) has been identified as the primary (if not only) cause of

- 2 -

blood-associated NANBH (BB-NANBH). See for example, PCT WO89/046699; U.S. Patent Application Serial No. 7/456,637, filed 21 December 1989; and U.S. Patent Application Serial No. 7/456,637, filed 21 December 1989, incorporated herein by reference. Hepatitis C appears to be the major form of transfusion-associated hepatitis
5 in a number of countries, including the United States and Japan. There is also evidence implicating HCV in induction of hepatocellular carcinoma. Thus, a need exists for an effective method for treating HCV infection: currently, there is none.

Many viruses, including adenoviruses, baculoviruses, comoviruses, picornaviruses, retroviruses, and togaviruses, rely on specific, virally-encoded proteases
10 for processing polypeptides from their initial translated form into mature, active proteins. In the case of picornaviruses, all of the viral proteins are believed to arise from cleavage of a single polyprotein (B.D. Korant, CRC Crit Rev Biotech (1988) 8:149-57).

S. Pichuantes et al, in "Viral Proteinases As Targets For Chemotherapy"
15 (Cold Spring Harbor Laboratory Press, 1989) pp. 215-22, disclosed expression of a viral protease found in HIV-1. The HIV protease was obtained in the form of a fusion protein, by fusing DNA encoding an HIV protease precursor to DNA encoding human superoxide dismutase (hSOD), and expressing the product in *E. coli*. Transformed cells expressed products of 36 and 10 kDa (corresponding to the hSOD-protease fusion
20 protein and the protease alone), suggesting that the protease was expressed in a form capable of autocatalytic proteolysis.

T.J. McQuade et al, Science (1990) 247:454-56 disclosed preparation of a peptide mimic capable of specifically inhibiting the HIV-1 protease. In HIV, the protease is believed responsible for cleavage of the initial p55 gag precursor transcript
25 into the core structural proteins (p17, p24, p8, and p7). Adding 1 μ M inhibitor to HIV-infected peripheral blood lymphocytes in culture reduced the concentration of processed HIV p24 by about 70%. Viral maturation and levels of infectious virus were reduced by the protease inhibitor.

- 3 -

Disclosure of the Invention

We have now invented recombinant HCV protease, HCV protease fusion proteins, truncated and altered HCV proteases, cloning and expression vectors therefore, and methods for identifying antiviral agents effective for treating HCV.

5

Brief Description of the Drawings

Figure 1 shows the sequence of HCV protease.

Figure 2 shows the polynucleotide sequence and deduced amino acid sequence of the clone C20c.

10 Figure 3 shows the polynucleotide sequence and deduced amino acid sequence of the clone C26d.

Figure 4 shows the polynucleotide sequence and deduced amino acid sequence of the clone C8h.

15 Figure 5 shows the polynucleotide sequence and deduced amino acid sequence of the clone C7f.

Figure 6 shows the polynucleotide sequence and deduced amino acid sequence of the clone C31.

Figure 7 shows the polynucleotide sequence and deduced amino acid sequence of the clone C35.

20 Figure 8 shows the polynucleotide sequence and deduced amino acid sequence of the clone C33c.

Figure 9 schematically illustrates assembly of the vector C7fC20cC300C200.

Figure 10 shows the sequence for cf1SODp600.

25 Modes of Carrying Out The Invention

A. Definitions

- 4 -

The terms "Hepatitis C Virus" and "HCV" refer to the viral species that is the major etiological agent of BB-NANBH, the prototype isolate of which is identified in PCT WO89/046699; EPO publication 318,216; USSN 7/355,008, filed 18 May 1989; and USSN 7/456,637, the disclosures of which are incorporated herein by reference. "HCV" as used herein includes the pathogenic strains capable of causing hepatitis C, and attenuated strains or defective interfering particles derived therefrom. The HCV genome is comprised of RNA. It is known that RNA-containing viruses have relatively high rates of spontaneous mutation, reportedly on the order of 10^{-3} to 10^{-4} per incorporated nucleotide (Fields & Knipe, "Fundamental Virology" (1986, Raven Press, N.Y.)). As heterogeneity and fluidity of genotype are inherent characteristics of RNA viruses, there will be multiple strains/isolates, which may be virulent or avirulent, within the HCV species.

Information on several different strains/isolates of HCV is disclosed herein, particularly strain or isolate CDC/HCVI (also called HCV1). Information from one strain or isolate, such as a partial genomic sequence, is sufficient to allow those skilled in the art using standard techniques to isolate new strains/isolates and to identify whether such new strains/isolates are HCV. For example, several different strains/isolates are described below. These strains, which were obtained from a number of human sera (and from different geographical areas), were isolated utilizing the information from the genomic sequence of HCV1.

The information provided herein suggests that HCV may be distantly related to the flaviviridae. The Flavivirus family contains a large number of viruses which are small, enveloped pathogens of man. The morphology and composition of Flavivirus particles are known, and are discussed in M.A. Brinton, in "The Viruses: The Togaviridae And Flaviviridae" (Series eds. Fraenkel-Conrat and Wagner, vol. eds. Schlesinger and Schlesinger, Plenum Press, 1986), pp. 327-374. Generally, with respect to morphology, Flaviviruses contain a central nucleocapsid surrounded by a lipid bilayer. Virions are spherical and have a diameter of about 40-50 nm. Their

- 5 -

cores are about 25-30 nm in diameter. Along the outer surface of the virion envelope are projections measuring about 5-10 nm in length with terminal knobs about 2 nm in diameter. Typical examples of the family include Yellow Fever virus, West Nile virus, and Dengue Fever virus. They possess positive-stranded RNA genomes (about 11,000 nucleotides) that are slightly larger than that of HCV and encode a polyprotein precursor of about 3500 amino acids. Individual viral proteins are cleaved from this precursor polypeptide.

The genome of HCV appears to be single-stranded RNA containing about 10,000 nucleotides. The genome is positive-stranded, and possesses a continuous translational open reading frame (ORF) that encodes a polyprotein of about 3,000 amino acids. In the ORF, the structural proteins appear to be encoded in approximately the first quarter of the N-terminal region, with the majority of the polyprotein attributed to non-structural proteins. When compared with all known viral sequences, small but significant co-linear homologies are observed with the non-structural proteins of the Flavivirus family, and with the pestiviruses (which are now also considered to be part of the Flavivirus family).

A schematic alignment of possible regions of a flaviviral polyprotein (using Yellow Fever Virus as an example), and of a putative polyprotein encoded in the major ORF of the HCV genome, is shown in Figure 1. Possible domains of the HCV polyprotein are indicated in the figure. The Yellow Fever Virus polyprotein contains, from the amino terminus to the carboxy terminus, the nucleocapsid protein (C), the matrix protein (M), the envelope protein (E), and the non-structural proteins 1, 2 (a+b), 3, 4 (a+b), and 5 (NS1, NS2, NS3, NS4, and NS5). Based upon the putative amino acids encoded in the nucleotide sequence of HCV1, a small domain at the extreme N-terminus of the HCV polyprotein appears similar both in size and high content of basic residues to the nucleocapsid protein (C) found at the N-terminus of flaviviral polyproteins. The non-structural proteins 2,3,4, and 5 (NS2-5) of HCV and of yellow fever virus (YFV) appear to have counterparts of similar size and hydropath-

- 6 -

icity, although the amino acid sequences diverge. However, the region of HCV which would correspond to the regions of YFV polyprotein which contains the M, E, and NS1 protein not only differs in sequence, but also appears to be quite different in size and hydropathicity. Thus, while certain domains of the HCV genome may be referred to herein as, for example, NS1, or NS2, it should be understood that these designations are for convenience of reference only; there may be considerable differences between the HCV family and flaviviruses that have yet to be appreciated.

Due to the evolutionary relationship of the strains or isolates of HCV, putative HCV strains and isolates are identifiable by their homology at the polypeptide level. With respect to the isolates disclosed herein, new HCV strains or isolates are expected to be at least about 40% homologous, some more than about 70% homologous, and some even more than about 80% homologous: some may be more than about 90% homologous at the polypeptide level. The techniques for determining amino acid sequence homology are known in the art. For example, the amino acid sequence may be determined directly and compared to the sequences provided herein. Alternatively the nucleotide sequence of the genomic material of the putative HCV may be determined (usually via a cDNA intermediate), the amino acid sequence encoded therein can be determined, and the corresponding regions compared.

The term "HCV protease" refers to an enzyme derived from HCV which exhibits proteolytic activity, specifically the polypeptide encoded in the NS3 domain of the HCV genome. At least one strain of HCV contains a protease believed to be substantially encoded by or within the following sequence:

25	Arg Arg Gly Arg Glu Ile Leu Leu Gly Pro	10
	Ala Asp Gly Met Val Ser Lys Gly Trp Arg	20
	Leu Leu Ala Pro Ile Thr Ala Tyr Ala Gln	30
	Gln Thr Arg Gly Leu Leu Gly Cys Ile Ile	40
	Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln	50
	Val Glu Gly Glu Val Gln Ile Val Ser Thr	60
30	Ala Ala Gln Thr Phe Leu Ala Thr Cys Ile	70
	Asn Gly Val Cys Trp Thr Val Tyr <u>His</u> Gly	80

- 7 -

Ala Gly Thr Arg Thr Ile Ala Ser Pro Lys 90
 Gly Pro Val Ile Gln Met Tyr Thr Asn Val 100
 Asp Gln Asp Leu Val Gly Trp Pro Ala Ser 110
 Gln Gly Thr Arg Ser Leu Thr Pro Cys Thr 120
 5 Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr 130
 Arg His Ala Asp Val Ile Pro Val Arg Arg 140
 Arg Gly Asp Ser Arg Gly Ser Leu Leu Ser 150
 Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser 160
Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly 170
 10 His Ala Val Gly Ile Phe Arg Ala Ala Val 180
 Cys Thr Arg Gly Val Ala Lys Ala Val Asp 190
 Phe Ile Pro Val Glu Asn Leu Glu Thr Thr 200
 Met Arg *** 202

The above N and C termini are putative, the actual termini being
 15 defined by expression and processing in an appropriate host of a DNA construct
 encoding the entire NS3 domain. It is understood that this sequence may vary from
 strain to strain, as RNA viruses like HCV are known to exhibit a great deal of
 variation. Further, the actual N and C termini may vary, as the protease is cleaved
 from a precursor polyprotein: variations in the protease amino acid sequence can
 20 result in cleavage from the polyprotein at different points. Thus, the amino- and
 carboxy-termini may differ from strain to strain of HCV. The first amino acid shown
 above corresponds to residue 60 in Figure 1. However, the minimum sequence
 necessary for activity can be determined by routine methods. The sequence may be
 truncated at either end by treating an appropriate expression vector with an exonucle-
 25 ase (after cleavage at the 5' or 3' end of the coding sequence) to remove any desired
 number of base pairs. The resulting coding polynucleotide is then expressed and the
 sequence determined. In this manner the activity of the resulting product may be
 correlated with the amino acid sequence: a limited series of such experiments
 (removing progressively greater numbers of base pairs) determines the minimum
 30 internal sequence necessary for protease activity. We have found that the sequence
 may be substantially truncated, particularly at the carboxy terminus, apparently with
 full retention of protease activity. It is presently believed that a portion of the protein

- 8 -

at the carboxy terminus may exhibit helicase activity. However, helicase activity is not required of the HCV proteases of the invention. The amino terminus may also be truncated to a degree without loss of protease activity.

The amino acids underlined above are believed to be the residues necessary for catalytic activity, based on sequence homology to putative flavivirus serine proteases. Table 1 shows the alignment of the three serine protease catalytic residues for HCV protease and the protease obtained from Yellow Fever Virus, West Nile Fever virus, Murray Valley Fever virus, and Kunjin virus. Although the other four flavivirus protease sequences exhibit higher homology with each other than with HCV, a degree of homology is still observed with HCV. This homology, however, was not sufficient for indication by currently available alignment software. The indicated amino acids are numbered His₇₉, Asp₁₀₃, and Ser₁₆₁ in the sequence listed above (His₁₃₉, Asp₁₆₃, and Ser₂₂₁ in Figure 1).

TABLE 1: Alignment of Active Residues by Sequence

Protease	His	Asp	Ser
HCV	CWTVY <u>H</u> GAG	DQDLGWPAP	LKGSSGGPL
Yellow Fever	FHTMWHVTR	KEDLVAYGG	PSGT <u>S</u> GSPI
West Nile Fever	FHTLW <u>H</u> TK	KEDRLCYGG	PTGT <u>S</u> GSPI
Murray Valley	FHTLW <u>H</u> TR	KEDRVTYGG	PIGT <u>S</u> GSPI
Kunjin Virus	FHTLW <u>H</u> TK	KEDRLCYGG	PTGT <u>S</u> GSPI

Alternatively, one can make catalytic residue assignments based on structural homology. Table 2 shows alignment of HCV with against the catalytic sites of several well-characterized serine proteases based on structural considerations:

protease A from *Streptomyces griseus*, α -lytic protease, bovine trypsin, chymotrypsin, and elastase (M. James et al, Can J Biochem (1978) 56:396). Again, a degree of

homology is observed. The HCV residues identified are numbered His₇₉, Asp₁₂₅, and Ser₁₆₁ in the sequence listed above.

TABLE 2: Alignment of Active Residues by Structure

Protease	His	Asp	Ser
<i>S. griseus</i> A	TAGHC	NNDYGH	GD\$GGSL
α -Lytic protease	TAGHC	GNDRAWV	GD\$GGSW
Bovine Trypsin	SAAHC	NNDIMLI	GD\$GGPV
Chymotrypsin	TAAHC	NNDITLL	GD\$GGPL
Elastase	TAAHC	GYDIAL	GD\$GGPL
HCV	TVYHG	SSDLYL	GSS\$GGPL

The most direct manner to verify the residues essential to the active site is to replace each residue individually with a residue of equivalent steric size. This is easily accomplished by site-specific mutagenesis and similar methods known in the art. If replacement of a particular residue with a residue of equivalent size results in loss of activity, the essential nature of the replaced residue is confirmed.

"HCV protease analogs" refer to polypeptides which vary from the full length protease sequence by deletion, alteration and/or addition to the amino acid sequence of the native protease. HCV protease analogs include the truncated proteases described above, as well as HCV protease muteins and fusion proteins comprising HCV protease, truncated protease, or protease muteins. Alterations to form HCV protease muteins are preferably conservative amino acid substitutions, in which an amino acid is replaced with another naturally-occurring amino acid of similar character. For example, the following substitutions are considered "conservative":

Gly \leftrightarrow Ala;	Lys \leftrightarrow Arg;
Val \leftrightarrow Ile \leftrightarrow Leu;	Asn \leftrightarrow Gln; and
Asp \leftrightarrow Glu;	Phe \leftrightarrow Trp \leftrightarrow Tyr.

- 10 -

Nonconservative changes are generally substitutions of one of the above amino acids with an amino acid from a different group (e.g., substituting Asn for Glu), or substituting Cys, Met, His, or Pro for any of the above amino acids. Substitutions involving common amino acids are conveniently performed by site specific
5 mutagenesis of an expression vector encoding the desired protein, and subsequent expression of the altered form. One may also alter amino acids by synthetic or semi-synthetic methods. For example, one may convert cysteine or serine residues to selenocysteine by appropriate chemical treatment of the isolated protein. Alternatively, one may incorporate uncommon amino acids in standard *in vitro* protein synthetic
10 methods. Typically, the total number of residues changed, deleted or added to the native sequence in the muteins will be no more than about 20, preferably no more than about 10, and most preferably no more than about 5.

The term fusion protein generally refers to a polypeptide comprising an amino acid sequence drawn from two or more individual proteins. In the present
15 invention, "fusion protein" is used to denote a polypeptide comprising the HCV protease, truncate, mutein or a functional portion thereof, fused to a non-HCV protein or polypeptide ("fusion partner"). Fusion proteins are most conveniently produced by expression of a fused gene, which encodes a portion of one polypeptide at the 5' end and a portion of a different polypeptide at the 3' end, where the different portions are
20 joined in one reading frame which may be expressed in a suitable host. It is presently preferred (although not required) to position the HCV protease or analog at the carboxy terminus of the fusion protein, and to employ a functional enzyme fragment at the amino terminus. As the HCV protease is normally expressed within a large polypeptide, it is not expected to include cell transport signals (e.g., export or secretion
25 signals). Suitable functional enzyme fragments are those polypeptides which exhibit a quantifiable activity when expressed fused to the HCV protease. Exemplary enzymes include, without limitation, β -galactosidase (β -gal), β -lactamase, horseradish peroxidase (HRP), glucose oxidase (GO), human superoxide dismutase (hSOD), urease,

- 11 -

and the like. These enzymes are convenient because the amount of fusion protein produced can be quantified by means of simple colorimetric assays. Alternatively, one may employ antigenic proteins or fragments, to permit simple detection and quantification of fusion proteins using antibodies specific for the fusion partner. The presently preferred fusion partner is hSOD.

B. General Method

The practice of the present invention generally employs conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See for example J. Sambrook et al, "Molecular Cloning; A Laboratory Manual (1989); "DNA Cloning", Vol. I and II (D.N Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed, 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. 1984); "Transcription And Translation" (B.D. Hames & S.J. Higgins eds. 1984); "Animal Cell Culture" (R.L Freshney ed. 1986); "Immobilized Cells And Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide To Molecular Cloning" (1984); the series, "Methods In Enzymology" (Academic Press, Inc.); "Gene Transfer Vectors For Mammalian Cells" (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory); Meth Enzymol (1987) 154 and 155 (Wu and Grossman, and Wu, eds., respectively); Mayer & Walker, eds. (1987), "Immunochemical Methods In Cell And Molecular Biology" (Academic Press, London); Scopes, "Protein Purification: Principles And Practice", 2nd Ed (Springer-Verlag, N.Y., 1987); and "Handbook Of Experimental Immunology", volumes I-IV (Weir and Blackwell, eds, 1986).

Both prokaryotic and eukaryotic host cells are useful for expressing desired coding sequences when appropriate control sequences compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing

- 12 -

operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers.

5 These plasmids are commercially available. The markers may be used to obtain successful transformants by selection. Commonly used prokaryotic control sequences include the β -lactamase (penicillinase) and lactose promoter systems (Chang et al, Nature (1977) 198:1056), the tryptophan (trp) promoter system (Goeddel et al, Nuc Acids Res (1980) 8:4057) and the lambda-derived P_L promoter and N gene ribosome
10 binding site (Shimatake et al, Nature (1981) 292:128) and the hybrid tac promoter (De Boer et al, Proc Nat Acad Sci USA (1983) 292:128) derived from sequences of the trp and lac UV5 promoters. The foregoing systems are particularly compatible with *E. coli*; if desired, other prokaryotic hosts such as strains of *Bacillus* or *Pseudomonas* may be used, with corresponding control sequences.

15 Eukaryotic hosts include without limitation yeast and mammalian cells in culture systems. Yeast expression hosts include *Saccharomyces*, *Klebsiella*, *Picia*, and the like. *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* and *K. lactis* are the most commonly used yeast hosts, and are convenient fungal hosts. Yeast-compatible vectors carry markers which permit selection of successful transformants by conferring prototrophy to auxotrophic mutants or resistance to heavy metals
20 on wild-type strains. Yeast compatible vectors may employ the 2 μ origin of replication (Broach et al, Meth Enzymol (1983) 101:307), the combination of CEN3 and ARS1 or other means for assuring replication, such as sequences which will result in incorporation of an appropriate fragment into the host cell genome. Control
25 sequences for yeast vectors are known in the art and include promoters for the synthesis of glycolytic enzymes (Hess et al, J Adv Enzyme Reg (1968) 7:149; Holland et al, Biochem (1978), 17:4900), including the promoter for 3-phosphoglycerate kinase (R. Hitzeman et al, J Biol Chem (1980) 255:2073). Terminators

- 13 -

may also be included, such as those derived from the enolase gene (Holland, J Biol Chem (1981) 256:1385). Particularly useful control systems are those which comprise the glyceraldehyde-3 phosphate dehydrogenase (GAPDH) promoter or alcohol dehydrogenase (ADH) regulatable promoter, terminators also derived from GAPDH, and if secretion is desired, a leader sequence derived from yeast α -factor (see U.S. Pat. No. 4,870,008, incorporated herein by reference).

A presently preferred expression system employs the ubiquitin leader as the fusion partner. Copending application USSN 7/390,599 filed 7 August 1989 disclosed vectors for high expression of yeast ubiquitin fusion proteins. Yeast ubiquitin provides a 76 amino acid polypeptide which is automatically cleaved from the fused protein upon expression. The ubiquitin amino acid sequence is as follows:

Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Thr
Leu Glu Val Glu Ser Ser Asp Thr Ile Asp Asn Val Lys
Ser Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp Gln
Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Asp Gly
Arg Thr Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser Thr
Leu His Leu Val Leu Arg Leu Arg Gly Gly

See also Ozkaynak et al, Nature (1984) 312:663-66. Polynucleotides encoding the ubiquitin polypeptide may be synthesized by standard methods, for example following the technique of Barr et al, J Biol Chem (1988) 268:1671-78 using an Applied Biosystem 380A DNA synthesizer. Using appropriate linkers, the ubiquitin gene may be inserted into a suitable vector and ligated to a sequence encoding the HCV protease or a fragment thereof.

In addition, the transcriptional regulatory region and the transcriptional initiation region which are operably linked may be such that they are not naturally associated in the wild-type organism. These systems are described in detail in EPO 120,551, published October 3, 1984; EPO 116,201, published August 22, 1984; and EPO 164,556, published December 18, 1985, all of which are commonly owned with the present invention, and are hereby incorporated herein by reference in full.

- 14 -

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including HeLa cells, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, and a number of other cell lines. Suitable promoters for mammalian cells are also known in the art and include viral promoters such as that from Simian Virus 40 (SV40) (Fiers et al, Nature (1978) 273:113), Rous sarcoma virus (RSV), adenovirus (ADV), and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly-A addition sequences. Enhancer sequences which increase expression may also be included, and sequences which promote amplification of the gene may also be desirable (for example methotrexate resistance genes). These sequences are known in the art.

Vectors suitable for replication in mammalian cells are known in the art, and may include viral replicons, or sequences which insure integration of the appropriate sequences encoding HCV epitopes into the host genome. For example, another vector used to express foreign DNA is Vaccinia virus. In this case the heterologous DNA is inserted into the Vaccinia genome. Techniques for the insertion of foreign DNA into the vaccinia virus genome are known in the art, and may utilize, for example, homologous recombination. The heterologous DNA is generally inserted into a gene which is non-essential to the virus, for example, the thymidine kinase gene (tk), which also provides a selectable marker. Plasmid vectors that greatly facilitate the construction of recombinant viruses have been described (see, for example, Mackett et al, J Virol (1984) 49:857; Chakrabarti et al, Mol Cell Biol (1985) 5:3403; Moss, in GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (Miller and Calos, eds., Cold Spring Harbor Laboratory, NY, 1987), p. 10). Expression of the HCV polypeptide then occurs in cells or animals which are infected with the live recombinant vaccinia virus.

In order to detect whether or not the HCV polypeptide is expressed from the vaccinia vector, BSC 1 cells may be infected with the recombinant vector

- 15 -

and grown on microscope slides under conditions which allow expression. The cells may then be acetone-fixed, and immunofluorescence assays performed using serum which is known to contain anti-HCV antibodies to a polypeptide(s) encoded in the region of the HCV genome from which the HCV segment in the recombinant expression vector was derived.

Other systems for expression of eukaryotic or viral genomes include insect cells and vectors suitable for use in these cells. These systems are known in the art, and include, for example, insect expression transfer vectors derived from the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV), which is a helper-independent, viral expression vector. Expression vectors derived from this system usually use the strong viral polyhedrin gene promoter to drive expression of heterologous genes. Currently the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373 (see PCT WO89/046699 and USSN 7/456,637). Many other vectors known to those of skill in the art have also been designed for improved expression. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and introduces a BamHI cloning site 32 bp downstream from the ATT; See Luckow and Summers, *Virology* (1989) 17:31). AcNPV transfer vectors for high level expression of nonfused foreign proteins are described in copending applications PCT WO89/046699 and USSN 7/456,637. A unique BamHI site is located following position -8 with respect to the translation initiation codon ATG of the polyhedrin gene. There are no cleavage sites for SmaI, PstI, BglII, XbaI or SstI. Good expression of nonfused foreign proteins usually requires foreign genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. The plasmid also contains the polyhedrin polyadenylation signal and the ampicillin-resistance (amp) gene and origin of replication for selection and propagation in *E. coli*.

Methods for the introduction of heterologous DNA into the desired site in the baculovirus virus are known in the art. (See Summer and Smith, Texas

- 16 -

Agricultural Experiment Station Bulletin No. 1555; Smith et al, Mol Cell Biol (1983) 3:2156-2165; and Luckow and Summers, Virology (1989) 17:31). For example, the heterologous DNA can be inserted into a gene such as the polyhedrin gene by homologous recombination, or into a restriction enzyme site engineered into the desired baculovirus gene. The inserted sequences may be those which encode all or varying segments of the polyprotein, or other orfs which encode viral polypeptides. For example, the insert could encode the following numbers of amino acid segments from the polyprotein: amino acids 1-1078; amino acids 332-662; amino acids 406-662; amino acids 156-328, and amino acids 199-328.

The signals for post-translational modifications, such as signal peptide cleavage, proteolytic cleavage, and phosphorylation, appear to be recognized by insect cells. The signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate cells and vertebrate cells. Examples of the signal sequences from vertebrate cells which are effective in invertebrate cells are known in the art, for example, the human interleukin-2 signal (IL₂) which signals for secretion from the cell, is recognized and properly removed in insect cells.

Transformation may be by any known method for introducing polynucleotides into a host cell, including, for example packaging the polynucleotide in a virus and transducing a host cell with the virus, and by direct uptake of the polynucleotide. The transformation procedure used depends upon the host to be transformed. Bacterial transformation by direct uptake generally employs treatment with calcium or rubidium chloride (Cohen, Proc Nat Acad Sci USA (1972) 69:2110; T. Maniatis et al, "Molecular Cloning; A Laboratory Manual" (Cold Spring Harbor Press, Cold Spring Harbor, NY, 1982). Yeast transformation by direct uptake may be carried out using the method of Hinnen et al, Proc Nat Acad Sci USA (1978) 75:1929. Mammalian transformations by direct uptake may be conducted using the calcium phosphate precipitation method of Graham and Van der Eb, Virology (1978) 52:546, or the various

- 17 -

known modifications thereof. Other methods for introducing recombinant polynucleotides into cells, particularly into mammalian cells, include dextran-mediated transfection, calcium phosphate mediated transfection, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the polynucleotides into nuclei.

Vector construction employs techniques which are known in the art. Site-specific DNA cleavage is performed by treating with suitable restriction enzymes under conditions which generally are specified by the manufacturer of these commercially available enzymes. In general, about 1 µg of plasmid or DNA sequence is cleaved by 1 unit of enzyme in about 20 µL buffer solution by incubation for 1-2 hr at 37°C. After incubation with the restriction enzyme, protein is removed by phenol/chloroform extraction and the DNA recovered by precipitation with ethanol. The cleaved fragments may be separated using polyacrylamide or agarose gel electrophoresis techniques, according to the general procedures described in Meth Enzymol (1980) 65:499-560.

Sticky-ended cleavage fragments may be blunt ended using *E. coli* DNA polymerase I (Klenow fragment) with the appropriate deoxynucleotide triphosphates (dNTPs) present in the mixture. Treatment with S1 nuclease may also be used, resulting in the hydrolysis of any single stranded DNA portions.

Ligations are carried out under standard buffer and temperature conditions using T4 DNA ligase and ATP; sticky end ligations require less ATP and less ligase than blunt end ligations. When vector fragments are used as part of a ligation mixture, the vector fragment is often treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase to remove the 5'-phosphate, thus preventing religation of the vector. Alternatively, restriction enzyme digestion of unwanted fragments can be used to prevent ligation.

- 18 -

Ligation mixtures are transformed into suitable cloning hosts, such as *E. coli*, and successful transformants selected using the markers incorporated (e.g., antibiotic resistance), and screened for the correct construction.

Synthetic oligonucleotides may be prepared using an automated
5 oligonucleotide synthesizer as described by Warner, DNA (1984) 3:401. If desired, the synthetic strands may be labeled with ^{32}P by treatment with polynucleotide kinase in the presence of ^{32}P -ATP under standard reaction conditions.

DNA sequences, including those isolated from cDNA libraries, may be modified by known techniques, for example by site directed mutagenesis (see e.g.,
10 Zoller, Nuc Acids Res (1982) 10:6487). Briefly, the DNA to be modified is packaged into phage as a single stranded sequence, and converted to a double stranded DNA with DNA polymerase, using as a primer a synthetic oligonucleotide complementary to the portion of the DNA to be modified, where the desired modification is included in the primer sequence. The resulting double stranded DNA is transformed into a phage-
15 supporting host bacterium. Cultures of the transformed bacteria which contain copies of each strand of the phage are plated in agar to obtain plaques. Theoretically, 50% of the new plaques contain phage having the mutated sequence, and the remaining 50% have the original sequence. Replicates of the plaques are hybridized to labeled synthetic probe at temperatures and conditions which permit hybridization with the
20 correct strand, but not with the unmodified sequence. The sequences which have been identified by hybridization are recovered and cloned.

DNA libraries may be probed using the procedure of Grunstein and Hogness Proc Nat Acad Sci USA (1975) 73:3961. Briefly, in this procedure the DNA to be probed is immobilized on nitrocellulose filters, denatured, and prehybridized with
25 a buffer containing 0-50% formamide, 0.75 M NaCl, 75 mM Na citrate, 0.02% (wt/v) each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll®, 50 mM NaH_2PO_4 (pH 6.5), 0.1% SDS, and 100 $\mu\text{g}/\text{mL}$ carrier denatured DNA. The percentage of formamide in the buffer, as well as the time and temperature conditions of the

prehybridization and subsequent hybridization steps depend on the stringency required. Oligomeric probes which require lower stringency conditions are generally used with low percentages of formamide, lower temperatures, and longer hybridization times. Probes containing more than 30 or 40 nucleotides, such as those derived from cDNA or genomic sequences generally employ higher temperatures, e.g., about 40-42°C, and a high percentage formamide, e.g., 50%. Following prehybridization, 5'-³²P-labeled oligonucleotide probe is added to the buffer, and the filters are incubated in this mixture under hybridization conditions. After washing, the treated filters are subjected to autoradiography to show the location of the hybridized probe; DNA in corresponding locations on the original agar plates is used as the source of the desired DNA.

For routine vector constructions, ligation mixtures are transformed into *E. coli* strain HB101 or other suitable hosts, and successful transformants selected by antibiotic resistance or other markers. Plasmids from the transformants are then prepared according to the method of Clewell et al, Proc Nat Acad Sci USA (1969) 62:1159, usually following chloramphenicol amplification (Clewell, J Bacteriol (1972) 110:667). The DNA is isolated and analyzed, usually by restriction enzyme analysis and/or sequencing. Sequencing may be performed by the dideoxy method of Sanger et al, Proc Nat Acad Sci USA (1977) 74:5463, as further described by Messing et al, Nuc Acids Res (1981) 9:309, or by the method of Maxam et al, Meth Enzymol (1980) 65:499. Problems with band compression, which are sometimes observed in GC-rich regions, were overcome by use of T-deazoguanosine according to Barr et al, Biotechniques (1986) 4:428.

The enzyme-linked immunosorbent assay (ELISA) can be used to measure either antigen or antibody concentrations. This method depends upon conjugation of an enzyme to either an antigen or an antibody, and uses the bound enzyme activity as a quantitative label. To measure antibody, the known antigen is fixed to a solid phase (e.g., a microtiter dish, plastic cup, dipstick, plastic bead, or the

- 20 -

like), incubated with test serum dilutions, washed, incubated with anti-immunoglobulin labeled with an enzyme, and washed again. Enzymes suitable for labeling are known in the art, and include, for example, horseradish peroxidase (HRP). Enzyme activity bound to the solid phase is usually measured by adding a specific substrate, and determining product formation or substrate utilization colorimetrically. The enzyme activity bound is a direct function of the amount of antibody bound.

To measure antigen, a known specific antibody is fixed to the solid phase, the test material containing antigen is added, after an incubation the solid phase is washed, and a second enzyme-labeled antibody is added. After washing, substrate is added, and enzyme activity is measured colorimetrically, and related to antigen concentration.

Proteases of the invention may be assayed for activity by cleaving a substrate which provides detectable cleavage products. As the HCV protease normally cleaves itself from the genomic polyprotein, one can employ this autocatalytic activity both to assay expression of the protein and determine activity. For example, if the protease is joined to its fusion partner so that the HCV protease N-terminal cleavage signal (Arg-Arg) is included, the expression product will cleave itself into fusion partner and active HCV protease. One may then assay the products, for example by western blot, to verify that the proteins produced correspond in size to the separate fusion partner and protease proteins. It is presently preferred to employ small peptide p-nitrophenyl esters or methylcoumarins, as cleavage may then be followed by spectrophotometric or fluorescent assays. Following the method described by E.D. Matayoshi et al, Science (1990) 247:231-35, one may attach a fluorescent label to one end of the substrate and a quenching molecule to the other end: cleavage is then determined by measuring the resulting increase in fluorescence. If a suitable enzyme or antigen has been employed as the fusion partner, the quantity of protein produced may easily be determined. Alternatively, one may exclude the HCV protease N-terminal cleavage signal (preventing self-cleavage) and add a separate cleavage sub-

- 21 -

strate, such as a fragment of the HCV NS3 domain including the native processing signal or a synthetic analog.

In the absence of this protease activity, the HCV polyprotein should remain in its unprocessed form, and thus render the virus noninfectious. Thus, the protease is useful for assaying pharmaceutical agents for control of HCV, as compounds which inhibit the protease activity sufficiently will also inhibit viral infectivity. Such inhibitors may take the form of organic compounds, particularly compounds which mimic the cleavage site of HCV recognized by the protease. Three of the putative cleavage sites of the HCV polyprotein have the following amino acid sequences:

Val-Ser-Ala-Arg-Arg // Gly-Arg-Glu-Ile-Leu-Leu-Gly
Ala-Ile-Leu-Arg-Arg // His-Val-Gly-Pro-
Val-Ser-Cys-Gln-Arg // Gly-Tyr-

15

These sites are characterized by the presence of two basic amino acids immediately before the cleavage site, and are similar to the cleavage sites recognized by other flavivirus proteases. Thus, suitable protease inhibitors may be prepared which mimic the basic/basic/small neutral motif of the HCV cleavage sites, but substituting a nonlabile linkage for the peptide bond cleaved in the natural substrate. Suitable inhibitors include peptide trifluoromethyl ketones, peptide boronic acids, peptide α -ketoesters, peptide difluoroketo compounds, peptide aldehydes, peptide diketones, and the like. For example, the peptide aldehyde N-acetyl-phenylalanyl-glycinaldehyde is a potent inhibitor of the protease papain. One may conveniently prepare and assay large mixtures of peptides using the methods disclosed in U.S. Patent application Serial No. 7/189,318, filed 2 May 1988 (published as PCT WO89/10931), incorporated herein by reference. This application teaches methods for generating mixtures of peptides up to hexapeptides having all possible amino acid

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- 22 -

sequences, and further teaches assay methods for identifying those peptides capable of binding to proteases.

Other protease inhibitors may be proteins, particularly antibodies and antibody derivatives. Recombinant expression systems may be used to generate
5 quantities of protease sufficient for production of monoclonal antibodies (MAbs) specific for the protease. Suitable antibodies for protease inhibition will bind to the protease in a manner reducing or eliminating the enzymatic activity, typically by obscuring the active site. Suitable MAbs may be used to generate derivatives, such as Fab fragments, chimeric antibodies, altered antibodies, univalent antibodies, and single
10 domain antibodies, using methods known in the art.

Protease inhibitors are screened using methods of the invention. In general, a substrate is employed which mimics the enzyme's natural substrate, but which provides a quantifiable signal when cleaved. The signal is preferably detectable by colorimetric or fluorometric means; however, other methods such as HPLC or
15 silica gel chromatography, GC-MS, nuclear magnetic resonance, and the like may also be useful. After optimum substrate and enzyme concentrations are determined, a candidate protease inhibitor is added to the reaction mixture at a range of concentrations. The assay conditions ideally should resemble the conditions under which the protease is to be inhibited *in vivo*, i.e., under physiologic pH, temperature,
20 ionic strength, etc. Suitable inhibitors will exhibit strong protease inhibition at concentrations which do not raise toxic side effects in the subject. Inhibitors which compete for binding to the protease active site may require concentrations equal to or greater than the substrate concentration, while inhibitors capable of binding irreversibly to the protease active site may be added in concentrations on the order of the
25 enzyme concentration.

In a presently preferred embodiment, an inactive protease mutein is employed rather than an active enzyme. It has been found that replacing a critical residue within the active site of a protease (e.g., replacing the active site Ser of a

- 23 -

serine protease) does not significantly alter the structure of the enzyme, and thus preserves the binding specificity. The altered enzyme still recognizes and binds to its proper substrate, but fails to effect cleavage. Thus, in one method of the invention an inactivated HCV protease is immobilized, and a mixture of candidate inhibitors added.

5 Inhibitors that closely mimic the enzyme's preferred recognition sequence will compete more successfully for binding than other candidate inhibitors. The poorly-binding candidates may then be separated, and the identity of the strongly-binding inhibitors determined. For example, HCV protease may be prepared substituting Ala for Ser₂₂₁ (Fig. 1), providing an enzyme capable of binding the HCV protease sub-
10 strate, but incapable of cleaving it. The resulting protease mutein is then bound to a solid support, for example Sephadex® beads, and packed into a column. A mixture of candidate protease inhibitors in solution is then passed through the column and fractions collected. The last fractions to elute will contain the strongest-binding compounds, and provide the preferred protease inhibitor candidates.

15 Protease inhibitors may be administered by a variety of methods, such as intravenously, orally, intramuscularly, intraperitoneally, bronchially, intranasally, and so forth. The preferred route of administration will depend upon the nature of the inhibitor. Inhibitors prepared as organic compounds may often be administered orally (which is generally preferred) if well absorbed. Protein-based inhibitors (such as most
20 antibody derivatives) must generally be administered by parenteral routes.

C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the
25 invention in any way.

- 24 -

Example 1

(Preparation of HCV cDNA)

A genomic library of HCV cDNA was prepared as described in PCT WO89/046699 and USSN 7/456,637. This library, ATCC accession no. 40394, has
5 been deposited as set forth below.

Example 2

(Expression of the Polypeptide Encoded in Clone 5-1-1.)

(A) The HCV polypeptide encoded within clone 5-1-1 (see
10 Example 1) was expressed as a fusion polypeptide with human superoxide dismutase (SOD). This was accomplished by subcloning the clone 5-1-1 cDNA insert into the expression vector pSODCF1 (K.S. Steimer et al, J Virol (1986) 58:9; EPO 138,111) as follows. The SOD/5-1-1 expression vector was transformed into *E. coli* D1210 cells. These cells, named Cf1/5-1-1 in *E. coli*, were deposited as set forth below and
15 have an ATCC accession no. of 67967.

First, DNA isolated from pSODCF1 was treated with BamHI and EcoRI, and the following linker was ligated into the linear DNA created by the restriction enzymes:

20 GAT CCT GGA ATT CTG ATA AGA CCT TAA GAC TAT TTT AA

After cloning, the plasmid containing the insert was isolated.

Plasmid containing the insert was restricted with EcoRI. The HCV cDNA insert in clone 5-1-1 was excised with EcoRI, and ligated into this EcoRI linearized plasmid DNA. The DNA mixture was used to transform *E. coli* strain D1210
5 (Sadler et al, Gene (1980) 8:279). Recombinants with the 5-1-1 cDNA in the correct orientation for expressing the ORF shown in Figure 1 were identified by restriction mapping and nucleotide sequencing.

Recombinant bacteria from one clone were induced to express the SOD-HCV₅₋₁₋₁ polypeptide by growing the bacteria in the presence of IPTG.

- 25 -

Three separate expression vectors, pcf1AB, pcf1CD, and pcf1EF were created by ligating three new linkers, AB, CD, and EF to a BamHI-EcoRI fragment derived by digesting to completion the vector pSODCF1 with EcoRI and BamHI, followed by treatment with alkaline phosphatase. The linkers were created from six oligomers, A, B, C, D, E, and F. Each oligomer was phosphorylated by treatment with kinase in the presence of ATP prior to annealing to its complementary oligomer. The sequences of the synthetic linkers were the following:

	Name	DNA Sequence (5' to 3')						
10	A	GATC	CTG	AAT	TCC	TGA	TAA	
	B		GAC	TTA	AGG	ACT	ATT TTA A	
	C	GATC	CGA	ATT	CTG	TGA	TAA	
	D		GCT	TAA	GAC	ACT	ATT TTA A	
15	E	GATC	CTG	GAA	TTC	TGA	TAA	
	F		GAC	CTT	AAG	ACT	ATT TTA A	

Each of the three linkers destroys the original EcoRI site, and creates a new EcoRI site within the linker, but within a different reading frame. Thus, the HCV cDNA EcoRI fragments isolated from the clones, when inserted into the expression vector, were in three different reading frames.

The HCV cDNA fragments in the designated λ gt11 clones were excised by digestion with EcoRI; each fragment was inserted into pcf1AB, pcf1CD, and pcf1EF. These expression constructs were then transformed into D1210 *E. coli* cells, the transformants cloned, and polypeptides expressed as described in part B below.

(B) Expression products of the indicated HCV cDNAs were tested for antigenicity by direct immunological screening of the colonies, using a modification of the method described in Helfman et al, Proc Nat Acad Sci USA (1983), 80:31. Briefly, the bacteria were plated onto nitrocellulose filters overlaid on ampicillin plates to give approximately 40 colonies per filter. Colonies were replica

- 26 -

plated onto nitrocellulose filters, and the replicas were regrown overnight in the presence of 2 mM IPTG and ampicillin. The bacterial colonies were lysed by suspending the nitrocellulose filters for about 15 to 20 min in an atmosphere saturated with CHCl_3 vapor. Each filter then was placed in an individual 100 mm Petri dish containing 10 mL of 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl_2 , 3% (w/v) BSA, 40 $\mu\text{g/mL}$ lysozyme, and 0.1 $\mu\text{g/mL}$ DNase. The plates were agitated gently for at least 8 hours at room temperature. The filters were rinsed in TBST (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.005% Tween® 20). After incubation, the cell residues were rinsed and incubated for one hour in TBS (TBST without Tween®) containing 10% sheep serum. The filters were then incubated with pretreated sera in TBS from individuals with NANBH, which included 3 chimpanzees; 8 patients with chronic NANBH whose sera were positive with respect to antibodies to HCV C100-3 polypeptide (also called C100); 8 patients with chronic NANBH whose sera were negative for anti-C100 antibodies; a convalescent patient whose serum was negative for anti-C100 antibodies; and 6 patients with community-acquired NANBH, including one whose sera was strongly positive with respect to anti-C100 antibodies, and one whose sera was marginally positive with respect to anti-C100 antibodies. The sera, diluted in TBS, was pretreated by preabsorption with hSOD for at least 30 minutes at 37°C. After incubation, the filters were washed twice for 30 min with TBST. The expressed proteins which bound antibodies in the sera were labeled by incubation for 2 hours with ^{125}I -labeled sheep anti-human antibody. After washing, the filters were washed twice for 30 min with TBST, dried, and autoradiographed.

Example 3

(Cloning of Full-Length SOD-Protease Fusion Proteins)

(A) pBR322-C200:

The nucleotide sequences of the HCV cDNAs used below were determined essentially as described above, except that the cDNA excised from these phages

- 27 -

were substituted for the cDNA isolated from clone 5-1-1.

Clone C33c was isolated using a hybridization probe having the following sequence:

5' ATC AGG ACC GGG GTG AGA ACA ATT ACC ACT 3'

- 5 The sequence of the HCV cDNA in clone C33c is shown in Figure 8, which also shows the amino acids encoded therein.

Clone 35 was isolated by screening with a synthetic polynucleotide having the sequence:

5' AAG CCA CCG TGT GCG CTA GGG CTC AAG CCC 3'

- 10 Approximately 1 in 50,000 clones hybridized with the probe. The polynucleotide and deduced amino acid sequences for C35 are shown in Figure 7.

- Clone C31 is shown in Figure 6, which also shows the amino acids encoded therein. A C200 cassette was constructed by ligating together a 718 bp fragment obtained by digestion of clone C33c DNA with EcoRI and HinfI, a 179 bp
15 fragment obtained by digestion of clone C31 DNA with HinfI and BglII, and a 377 bp fragment obtained by digesting clone C35 DNA with BglII and EcoRI. The construct of ligated fragments were inserted into the EcoRI site of pBR322, yielding the plasmid pBR322-C200.

(B) C7f+C20c:

- 20 Clone 7f was isolated using a probe having the sequence:

5'-AGC AGA CAA GGG GCC TCC TAG GGT GCA TAA T-3'

The sequence of HCV cDNA in clone 7f and the amino acids encoded therein are shown in Figure 5.

Clone C20c is isolated using a probe having the following sequence:

- 25 5'-TGC ATC AAT GGG GTG TGC TGG-3'

The sequence of HCV cDNA in clone C20c, and the amino acids encoded therein are shown in Figure 2.

- 28 -

Clones 7f and C20c were digested with EcoRI and SfaNI to form 400 bp and 260 bp fragments, respectively. The fragments were then cloned into the EcoRI site of pBR322 to form the vector C7f+C20c, and transformed into HB101 cells.

(C) C300:

5 Clone 8h was isolated using a probe based on the sequence of nucleotides in clone 33c. The nucleotide sequence of the probe was

5'-AGA GAC AAC CAT GAG GTC CCC GGT GTT C-3'.

The sequence of the HCV cDNA in clone 8h, and the amino acids encoded therein, are shown in Figure 4.

10 Clone C26d is isolated using a probe having the following sequence:

5'-CTG TTG TGC CCC GCG GCA GCC-3'

The sequence and amino acid translation of clone C26d is shown in Figure 3.

15 Clones C26d and C33c (see part A above) were transformed into the methylation minus *E. coli* strain GM48. Clone C26d was digested with EcoRII and DdeI to provide a 100 bp fragment. Clone C33c was digested with EcoRII and EcoRI to provide a 700 bp fragment. Clone C8h was digested with EcoRI and DdeI to provide a 208 bp fragment. These three fragments were then ligated into the EcoRI site of pBR322, and transformed into *E. coli* HB101, to provide the vector C300.

20 (D) Preparation of Full Length Clones:

A 600 bp fragment was obtained from C7f+C20c by digestion with EcoRI and NaeI, and ligated to a 945 bp NaeI/EcoRI fragment from C300, and the construct inserted into the EcoRI site of pGEM4Z (commercially available from Promega) to form the vector C7fC20cC300.

25 C7fC20cC300 was digested with NdeI and EcoRI to provide a 892 bp fragment, which was ligated with a 1160 bp fragment obtained by digesting C200 with NdeI and EcoRI. The resulting construct was inserted into the EcoRI site of pBR322

- 29 -

to provide the vector C7fC20cC300C200. Construction of this vector is illustrated schematically in Figure 9.

Example 4

(Preparation of *E. coli* Expression Vectors)

(A) cf1SODp600:

This vector contains a full-length HCV protease coding sequence fused to a functional hSOD leader. The vector C7fC20cC300C200 was cleaved with EcoRI to provide a 2000 bp fragment, which was then ligated into the EcoRI site of plasmid cf1CD (Example 2A). The resulting vector encodes amino acids 1-151 of hSOD, and amino acids 946-1630 of HCV (numbered from the beginning of the polyprotein, corresponding to amino acids 1-686 in Figure 1). The vector was labeled cf1SODp600 (sometimes referred to as P600), and was transformed into *E. coli* D1210 cells. These cells, ATCC accession no. 68275, were deposited as set forth below.

(B) P190:

A truncated SOD-protease fusion polynucleotide was prepared by excising a 600 bp EcoRI/NaeI fragment from C7f+C20c, blunting the fragment with Klenow fragment, ligating the blunted fragment into the Klenow-blunted EcoRI site of cf1EF (Example 2A). This polynucleotide encodes a fusion protein having amino acids 1-151 of hSOD, and amino acids 1-199 of HCV protease.

(C) P300:

A longer truncated SOD-protease fusion polynucleotide was prepared by excising an 892 bp EcoRI/NdeI fragment from C7fC20cC300, blunting the fragment with Klenow fragment, ligating the blunted fragment into the Klenow-blunted EcoRI site of cf1EF. This polynucleotide encodes a fusion protein having amino acids 1-151 of hSOD, and amino acids 1-299 of HCV protease.

- 30 -

(D) P500:

A longer truncated SOD-protease fusion polynucleotide was prepared by excising a 1550 bp EcoRI/EcoRI fragment from C7fC20cC300, and ligating the fragment into the EcoRI site of cf1CD to form P500. This polynucleotide encodes a fusion protein having amino acids 1-151 of hSOD, and amino acids 946-1457 of HCV protease (amino acids 1-513 in Figure 1).

(E) FLAG/Protease Fusion

This vector contains a full-length HCV protease coding sequence fused to the FLAG sequence, Hopp et al. (1988) Biotechnology 6: 1204-1210. PCR was used to produce a HCV protease gene with special restriction ends for cloning ease. Plasmid p500 was digested with EcoRI and NdeI to yield a 900 bp fragment. This fragment and two primers were used in a polymerase chain reaction to introduce a unique BglII site at amino acid 1009 and a stop codon with a SalI site at amino acid 1262 of the HCV-1, as shown in Figure 17 of WO 90/11089, published 4 October 1990. The sequence of the primers is as follows:

5' CCC GAG CAA GAT CTC CCG GCC C 3'

and

5' CCC GGC TGC ATA AGC AGT CGA CTT GGA 3'

After 30 cycles of PCR, the reaction was digested with BglII and SalI, and the 710 bp fragment was isolated. This fragment was annealed and ligated to the following duplex:

MetAspTyrLysAspAspAspAspLysGlyArgGlu
CATGGACTACAAAGACGATGACGATAAAGGCCGGGA
CTGATGTTTCTGCTACTGCTATTTCGGCCCTCTAG

The duplex encodes the FLAG sequence, and initiator methionine, and a 5' NcoI restriction site. The resulting NcoI/SalI fragment was ligated into a derivative of pCF1, which lacks the SOD gene and contains an optimized ribosome binding site for enhanced translational efficiency.

- 31 -

This construct is then transformed into *E. coli* D1210 cells and expression of the protease is induced by the addition of IPTG.

The FLAG sequence was fused to the HCV protease to facilitate purification. A calcium dependent monoclonal antibody, which binds to the FLAG encoded peptide,
5 is used to purify the fusion protein without harsh eluting conditions.

Example 5

(E. coli Expression of SOD-Protease Fusion Proteins)

(A) *E. coli* D1210 cells were transformed with cf1SODp600 and grown in
10 Luria broth containing 100 µg/mL ampicillin to an OD of 0.3-0.5. IPTG was then added to a concentration of 2 mM, and the cells cultured to a final OD of 0.9 to 1.3. The cells were then lysed, and the lysate analyzed by Western blot using anti-HCV sera, as described in USSN 7/456,637.

The results indicated the occurrence of cleavage, as no full length product
15 (theoretical Mr 93 kDa) was evident on the gel. Bands corresponding to the hSOD fusion partner and the separate HCV protease appeared at relative molecular weights of about 34, 53, and 66 kDa. The 34 kDa band corresponds to the hSOD partner (about 20 kDa) with a portion of the NS3 domain, while the 53 and 66 kDa bands correspond to HCV protease with varying degrees of (possibly bacterial) processing.

20 (B) *E. coli* D1210 cells were transformed with P500 and grown in Luria broth containing 100 µg/mL ampicillin to an OD of 0.3-0.5. IPTG was then added to a concentration of 2 mM, and the cells cultured to a final OD of 0.8 to 1.0. The cells were then lysed, and the lysate analyzed as described above.

The results again indicated the occurrence of cleavage, as no full length
25 product (theoretical Mr 73 kDa) was evident on the gel. Bands corresponding to the hSOD fusion partner and the truncated HCV protease appeared at molecular weights of about 34 and 45 kDa, respectively.

- 32 -

(C) *E. coli* D1210 cells were transformed with vectors P300 and P190 and grown as described above.

The results from P300 expression indicated the occurrence of cleavage, as no full length product (theoretical Mr 51 kDa) was evident on the gel. A band
5 corresponding to the hSOD fusion partner appeared at a relative molecular weight of about 34. The corresponding HCV protease band was not visible, as this region of the NS3 domain is not recognized by the sera employed to detect the products. However, appearance of the hSOD band at 34 kDa rather than 51 kDa indicates that cleavage occurred.

10 The P190 expression product appeared only as the full (encoded) length product without cleavage, forming a band at about 40 kDa, which corresponds to the theoretical molecular weight for the uncleaved product. This may indicate that the minimum essential sequence for HCV protease extends to the region between amino acids 199 and 299.

15

Example 6

(Purification of *E. coli* Expressed Protease)

The HCV protease and fragments expressed in Example 5 may be purified as follows:

20 The bacterial cells in which the polypeptide was expressed are subjected to osmotic shock and mechanical disruption, the insoluble fraction containing the protease is isolated and subjected to differential extraction with an alkaline-NaCl solution, and the polypeptide in the extract purified by chromatography on columns of S-Sepharose® and Q-Sepharose®.

25 The crude extract resulting from osmotic shock and mechanical disruption is prepared by suspending 1 g of the packed cells in 10 mL of a solution containing 0.02 M Tris HCl, pH 7.5, 10 mM EDTA, 20% sucrose, and incubating for 10 minutes on ice. The cells are then pelleted by centrifugation at 4,000 x g for 15 min at 4°C.

- 33 -

After the supernatant is removed, the cell pellets are resuspended in 10 mL of Buffer A1 (0.01 M Tris HCl, pH 7.5, 1 mM EDTA, 14 mM β -mercaptoethanol - " β ME"), and incubated on ice for 10 minutes. The cells are again pelleted at 4,000 x g for 15 minutes at 4°C. After removal of the clear supernatant (periplasmic fraction I), the cell pellets are resuspended in Buffer A1, incubated on ice for 10 minutes, and again centrifuged at 4,000 x g for 15 minutes at 4°C. The clear supernatant (periplasmic fraction II) is removed, and the cell pellet resuspended in 5 mL of Buffer T2 (0.02 M Tris HCl, pH 7.5, 14 mM β ME, 1 mM EDTA, 1 mM PMSF). In order to disrupt the cells, the suspension (5 mL) and 7.5 mL of Dyno-mill lead-free acid washed glass beads (0.10-0.15 mm diameter) (available from Glen-Mills, Inc.) are placed in a Falcon tube and vortexed at top speed for two minutes, followed by cooling for at least 2 min on ice. The vortexing-cooling procedure is repeated another four times. After vortexing, the slurry is filtered through a sintered glass funnel using low suction, the glass beads washed twice with Buffer A2, and the filtrate and washes combined.

15. The insoluble fraction of the crude extract is collected by centrifugation at 20,000 x g for 15 min at 4°C, washed twice with 10 mL Buffer A2, and resuspended in 5 mL of MILLI-Q water.

A fraction containing the HCV protease is isolated from the insoluble material by adding to the suspension NaOH (2 M) and NaCl (2 M) to yield a final concentration of 20 mM each, vortexing the mixture for 1 minute, centrifuging it 20,000 x g for 20 min at 4°C, and retaining the supernatant.

The partially purified protease is then purified by SDS-PAGE. The protease may be identified by western blot, and the band excised from the gel. The protease is then eluted from the band, and analyzed to confirm its amino acid sequence. N-terminal sequences may be analyzed using an automated amino acid sequencer, while C-terminal sequences may be analyzed by automated amino acid sequencing of a series of tryptic fragments.

- 34 -

Example 7

(Preparation of Yeast Expression Vector)

(A) P650 (SOD/Protease Fusion)

This vector contains HCV sequence, which includes the wild-type full-length HCV protease coding sequence, fused at the 5' end to a SOD coding sequence. Two fragments, a 441 bp EcoRI/BglII fragment from clone 11b and a 1471 bp BglII/EcoRI fragment from expression vector P500, were used to reconstruct a wild-type, full-length HCV protease coding sequence. These two fragments were ligated together with an EcoRI digested pS356 vector to produce an expression cassette. The expression cassette encodes the ADH2/GAPDH hybrid yeast promoter, human SOD, the HCV protease, and a GAPDH transcription terminator. The resulting vector was digested with BamHI and a 4052 bp fragment was isolated. This fragment was ligated to the BamHI digested pAB24 vector to produce p650. p650 expresses a polypeptide containing, from its amino terminal end, amino acids 1-154 of hSOD, an oligopeptide -Asn-Leu-Gly-Ile-Arg-, and amino acids 819 to 1458 of HCV-1, as shown in Figure 17 of WO 90/11089, published 4 October 1990.

Clone 11b was isolated from the genomic library of HCV cDNA, ATCC accession no. 40394, as described above in Example 3A, using a hybridization probe having the following sequence:

5' CAC CTA TGT TTA TAA CCA TCT CAC TCC TCT 3'.

This procedure is also described in EPO Pub. No. 318 216, Example IV.A.17.

The vector pS3EF, which is a pBR322 derivative, contains the ADH2/GAPDH hybrid yeast promoter upstream of the human superoxide dismutase gene, an adaptor, and a downstream yeast effective transcription terminator. A similar expression vector containing these control elements and the superoxide dismutase gene is described in Cousens et al. (1987) Gene 61: 265, and in copending application EPO 196,056, published October 1, 1986. pS3EF, however, differs from that in Cousens et al. in

- 35 -

that the heterologous proinsulin gene and the immunoglobulin hinge are deleted, and Gln₁₃₄ of SOD is followed by an adaptor sequence which contains an EcoRI site. The sequence of the adaptor is:

5' AAT TTG GGA ATT CCA TAA TTA ATT AAG 3'
3' AC CCT TAA GGT ATT AAT TAA TTC AGCT 5'

The EcoRI site facilitates the insertion of heterologous sequences. Once inserted into pS3EF, a SOD fusion is expressed which contains an oligopeptide that links SOD to the heterologous sequences. pS3EF is exactly the same as pS356 except that pS356 contains a different adaptor. The sequence of the adaptor is shown below:

5' AAT TTG GGA ATT CCA TAA TGA G 3'
3' AC CCT TAA GGT ATT ACT CAG CT 5'

pS356, ATCC accession no. 67683, is deposited as set forth below.

Plasmid pAB24 is a yeast shuttle vector, which contains pBR322 sequences, the complete 2 μ sequence for DNA replication in yeast (Broach (1981) in: Molecular Biology of the Yeast Saccharomyces, Vol. 1, p. 445, Cold spring Harbor Press.) and the yeast LEU^{2d} gene derived from plasmid pC1/1, described in EPO Pub. No. 116 201. Plasmid pAB24 was constructed by digesting YEp24 with EcoRI and re-ligating the vector to remove the partial 2 micron sequences. The resulting plasmid, YEp24deltaRI, was linearized with ClaI and ligated with the complete 2 micron plasmid which had been linearized with ClaI. The resulting plasmid, pCBou, was then digested with XbaI, and the 8605 bp vector fragment was gel isolated. This isolated XbaI fragment was ligated with a 4460 bp XbaI fragment containing the LEU^{2d} gene isolated from pC1/1; the orientation of LEU^{2d} gene is in the same direction as the URA3 gene.

S. cerevisiae, 2150-2-3 (pAB24-GAP-env2), accession no. 20827, is deposited with the American Type Culture Collection as set forth below. The plasmid pAB24-GAP-env2 can be recovered from the yeast cells by known techniques. The GAP-env2 expression cassette can be removed by digesting pAB24-GAP-env2 with BamHI. pAB24 is recovered by religating the vector without the BamHI insert.

- 36 -

Example 8

(Yeast Expression of SOD-Protease Fusion Protein)

p650 was transformed in *S. cerevisiae* strain JSC310, Mata, leu2, ura3-52, prb1-1122, pep4-3, prc1-407, cir^o: DM15 (g418 resistance). The transformation is as described by Hinnen et al. (1978) Proc Natl Acad Sci USA 75: 1929. The transformed cells were selected on ura- plates with 8% glucose. The plates were incubated at 30°C for 4-5 days. The transformants were further selected on leu- plates with 8% glucose putatively for high numbers of the p650 plasmid. Colonies from the leu- plates were inoculated into leu- medium with 3% glucose. These cultures were shaken at 30°C for 2 days and then diluted 1/20 into YEPD medium with 2% glucose and shaken for 2 more days at 30°C.

S. cerevisiae JSC310 contains DM15 DNA, described in EPO Pub. No. 340 986, published 8 November 1989. This DM15 DNA enhances ADH2 regulated expression of heterologous proteins. pDM15, accession no. 40453, is deposited with the American Type Culture Collection as set forth below.

Example 9

(Yeast Ubiquitin Expression of Mature HCV Protease)

Mature HCV protease is prepared by cleaving vector C7fC20cC300C200 with EcoRI to obtain a 2 Kb coding sequence, and inserting the sequence with the appropriate linkers into a ubiquitin expression vector, such as that described in WO 88/02406, published 7 April 1988, or USSN 7/390,599 filed 7 August 1989, incorporated herein by reference. Mature HCV protease is recovered upon expression of the vector in suitable hosts, particularly yeast. Specifically, the yeast expression protocol described in Example 8 is used to express a ubiquitin/HCV protease vector.

- 37 -

Example 10

(Preparation of an In-Vitro Expression Vector)

(A) pGEM®-3Z/Yellow Fever Leader Vector

5 Four synthetic DNA fragments were annealed and ligated** together to
create a EcoRI/SacI Yellow Fever leader, which was ligated to a EcoRI/SacI digested
pGEM®-3Z vector from Promega®. The sequence of the four fragments are listed
below:

YFK-1:

5' AAT TCG TAA ATC CTG TGT GCT AAT TGA GGT GCA TTG GTC TGC
10 AAA TCG AGT TGC TAG GCA ATA AAC ACA TT 3'

YFK-2:

5' TAT TGC CTA GCA ACT CGA TTT GCA GAC CAA TGC ACC TCA ATT
AGC ACA CAG GAT TTA CG 3'

YFK-3:

15 5' TGG ATT AAT TTT AAT CGT TCG TTG AGC GAT TAG CAG AGA ACT
GAC CAG AAC ATG TCT GAG CT 3'

YFK-4:

5' CAG ACA TGT TCT GGT CAG TTC TCT GCT AAT CGC TCA ACG AAC
GAT TAA AAT TAA TCC AAA TGT GTT 3'.

20 For in-vitro translation of the HCV protease, the new pGEM®-
3Z/Yellow Fever leader vector was digested with BamHI and blunted with Klenow.

(B) PvuII Construct from p6000

A clone p6000 was constructed from sequences available from the
genomic library of HCV cDNA, ATCC accession no. 40394. The HCV encoding
25 DNA sequence of p6000 is identical to nucleotide -275 to nucleotide 6372 of Figure
17 of WO 90/11089, published 4 October 1990. p6000 was digested with PvuII, and
from the digest, a 2,864 bp fragment was isolated. This 2,864 bp fragment was

- 38 -

ligated to the prepared pGEM®-3Z/Yellow Fever leader vector fragment, described above.

Example 11

5

(In-Vitro Expression of HCV Protease)

(A) Transcription

The pGEM®-3Z/Yellow Fever leader/PvuII vector was linearized with XbaI and transcribed using the materials and protocols from Promega's Riboprobe® Gemini II Core system.

10

(B) Translation

The RNA produced by the above protocol was translated using Promega's rabbit reticulocyte lysate, minus methionine, canine pancreatic microsomal membranes, as well as, other necessary materials and instructions from Promega.

15

Deposited Biological Materials:

The following materials were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville, Maryland:

20

<u>Name</u>	<u>Deposit Date</u>	<u>Accession No.</u>
<i>E. coli</i> D1210, cf1SODp600	23 Mar 1990	68275
Cf1/5-1-1 in <i>E. coli</i> D1210	11 May 1989	67967
25 Bacteriophage λ-gt11 cDNA library	01 Dec 1987	40394
<i>E. coli</i> HB101, pS356	29 Apr 1988	67683
30 plasmid DNA, pDM15	05 May 1988	40453
<i>S. cerevisiae</i> , 2150-2-3 (pAB24-GAP-env2)	23 Dec 1986	20827

- 39 -

The above materials have been deposited with the ATCC under the accession numbers indicated. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for purposes of Patent Procedure. These deposits are provided as a convenience to those of skill in the art, and are not an admission that a deposit is required under 35 U.S.C. §112. The polynucleotide sequences contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with the sequences described herein. A license may be required to make, use or sell the deposited materials, and no such license is granted hereby.

- 40 -

WHAT IS CLAIMED:

1. A method for assaying compounds for activity against Hepatitis C virus, which method comprises:
 - 5 providing a proteolytically inactive HCV protease analog;
 - contacting said inactive HCV protease analog with a mixture of candidate anti-HCV compounds; and
 - determining which candidate compounds bind to said HCV protease analog.
- 10 2. The method of claim 1, wherein said inactive HCV protease analog has substantially the following sequence:

Arg Arg Gly Arg Glu Ile Leu Leu Gly Pro Ala Asp Gly Met Val Ser
 Lys Gly Trp Arg Leu Leu Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr
 15 Arg Gly Leu Leu Gly Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys
 Asn Gln Val Glu Gly Glu Val Gln Ile Val Ser Thr Ala Ala Gln Thr Phe
 Leu Ala Thr Cys Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala
 Gly Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr Thr
 Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg
 20 Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr
 Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg Gly
 Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser Ala Gly Gly
 Pro Leu Leu Cys Pro Ala Gly His Ala Val Gly Ile Phe Arg Ala Ala Val
 Cys Thr Arg Gly Val Ala Lys Ala Val Asp Phe Ile Pro Val Glu Asn
 Leu Glu Thr Thr Met Arg.
- 25 3. The method of claim 1, wherein said inactive HCV protease analog has substantially the following sequence:

Gly Thr Tyr Val Tyr Asn His Leu Thr Pro Leu Arg Asp Trp Ala His
 Asn Gly Leu Arg Asp Leu Ala Val Ala Val Glu Pro Val Val Phe Ser
 30 Gln Met Glu Thr Lys Leu Ile Thr Trp Gly Ala Asp Thr Ala Ala Cys
 Gly Asp Ile Ile Asn Gly Leu Pro Val Ser Ala Arg Arg Gly Arg Glu Ile
 Leu Leu Gly Pro Ala Asp Gly Met Val Ser Lys Gly Trp Arg Leu Leu
 Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile
 Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu Val

Figure 1

			130					135					140				
Thr	Cys	Ile	Ile	Asn	Gly	Val	Cys	Trp	Thr	Val	Tyr	His	Gly	Ala	Gly		
ACG	TGC	ATC	ATC	AAT	GGG	GTG	TGC	TGG	ACT	GTC	TAC	CAC	GGG	GCC	GGA		
TGC	ACG	TAG	TAG	TTA	CCC	CAC	ACG	ACC	TGA	CAG	ATG	GTG	CCC	CGG	CCT		
		145						150					155				
Thr	Arg	Thr	Ile	Ala	Ser	Pro	Lys	Gly	Pro	Val	Ile	Gln	Met	Tyr	Thr		
ACG	AGG	ACC	ATC	GCG	TCA	CCC	AAG	GGT	CCT	GTC	ATC	CAG	ATG	TAT	ACC		
TGC	TCC	TGG	TAG	CGC	AGT	GGG	TTC	CCA	GGA	CAG	TAG	GTG	TAC	ATA	TGG		
	160						165					170					
Asn	Val	Asp	Gln	Asp	Leu	Val	Gly	Trp	Pro	Ala	Ser	Gln	Gly	Thr	Arg		
AAT	GTA	GAC	CAA	GAC	CTT	GTG	GGC	TGG	CCC	GCT	TCG	CAA	GGT	ACC	CGC		
TTA	CAT	CTG	GTT	CTG	GAA	CAC	CCG	ACC	GGG	CGA	AGC	GTT	CCA	TGG	GCG		
175						180					185				190		
Ser	Leu	Thr	Pro	Cys	Thr	Cys	Gly	Ser	Ser	Asp	Leu	Tyr	Leu	Val	Thr		
TCA	TTG	ACA	CCC	TGC	ACT	TGC	GGC	TCC	TCG	GAC	CTT	TAC	CTG	GTC	ACG		
AGT	AAC	TGT	GGG	ACG	TGA	ACG	CCG	AGG	AGC	CTG	GAA	ATG	GAC	CAG	TGC		
			195						200					205			
Arg	His	Ala	Asp	Val	Ile	Pro	Val	Arg	Arg	Arg	Gly	Asp	Ser	Arg	Gly		
AGG	CAC	GCC	GAT	GTC	ATT	CCC	GTG	CGC	CGG	CGG	GGT	GAT	AGC	AGG	GGC		
TCC	GTG	CGG	CTA	CAG	TAA	GGG	CAC	GCG	GCC	GCC	CCA	CTA	TCG	TCC	CCG		
								↑									
								NaeI									
		210						215					220				
Ser	Leu	Leu	Ser	Pro	Arg	Pro	Ile	Ser	Tyr	Leu	Lys	Gly	Ser	Ser	Gly		
AGC	CTG	CTG	TCG	CCC	CGG	CCC	ATT	TCC	TAC	TTG	AAA	GGC	TCC	TCG	GGG		
TCG	GAC	GAC	AGC	GGG	GCC	GGG	TAA	AGG	ATG	AAC	TTT	CCG	AGG	AGC	CCC		
	225						230					235					
Gly	Pro	Leu	Leu	Cys	Pro	Ala	Gly	His	Ala	Val	Gly	Ile	Phe	Arg	Ala		
GGT	CCG	CTG	TTG	TGC	CCC	GCG	GGG	CAC	GCC	GTG	GGC	ATA	TTT	AGG	GCC		
CCA	GGC	GAC	AAC	ACG	GGG	CGC	CCC	GTG	CGG	CAC	CCG	TAT	AAA	TCC	CGG		
240						245					250						
Ala	Val	Cys	Thr	Arg	Gly	Val	Ala	Lys	Ala	Val	Asp	Phe	Ile	Pro	Val		
GCG	GTG	TGC	ACC	CGT	GGA	GTG	GCT	AAG	GCG	GTG	GAC	TTT	ATC	CCT	GTG		
CGC	CAC	ACG	TGG	GCA	CCT	CAC	CGA	TTC	CGC	CAC	CTG	AAA	TAG	GGA	CAC		

Figure 1 (continued)

Figure 1 (continued)

480					485					490					
Val	Ser	Val	Ile	Pro	Thr	Ser	Gly	Asp	Val	Val	Val	Val	Ala	Thr	Asp
GTG	TCC	GTC	ATC	CCG	ACC	AGC	GGC	GAT	GTT	GTC	GTC	GTG	GCA	ACC	GAT
CAC	AGG	CAG	TAG	GGC	TGG	TCG	CCG	CTA	CAA	CAG	CAG	CAC	CGT	TGG	CTA

Figure 1 (continued)

495					500					505					510
Ala	Leu	Met	Thr	Gly	Tyr	Thr	Gly	Asp	Phe	Asp	Ser	Val	Ile	Asp	Cys
GCC	CTC	ATG	ACC	GGC	TAT	ACC	GGC	GAC	TTC	GAC	TCG	GTG	ATA	GAC	TGC
CGG	GAG	TAC	TGG	CCG	ATA	TGG	CCG	CTG	AAG	CTG	AGC	CAC	TAT	CTG	ACG

				515					520					525	
Asn	Thr	Cys	Val	Thr	Gln	Thr	Val	Asp	Phe	Ser	Leu	Asp	Pro	Thr	Phe
AAT	ACG	TGT	GTC	ACC	CAG	ACA	GTC	GAT	TTC	AGC	CTT	GAC	CCT	ACC	TTC
TTA	TGC	ACA	CAG	TGG	GTC	TGT	CAG	CTA	AAG	TCG	GAA	CTG	GGA	TGG	AAG

			530					535					540		
Thr	Ile	Glu	Thr	Ile	Thr	Leu	Pro	Gln	Asp	Ala	Val	Ser	Arg	Thr	Gln
ACC	ATT	GAG	ACA	ATC	ACG	CTC	CCC	CAA	GAT	GCT	GTC	TCC	CGC	ACT	CAA
TGG	TAA	CTC	TGT	TAG	TGC	GAG	GGG	GTT	CTA	CGA	CAG	AGG	GCG	TGA	GTT

		545					550					555			
Arg	Arg	Gly	Arg	Thr	Gly	Arg	Gly	Lys	Pro	Gly	Ile	Tyr	Arg	Phe	Val
CGT	CGG	GGC	AGG	ACT	GGC	AGG	GGG	AAG	CCA	GGC	ATC	TAC	AGA	TTT	GTG
GCA	GCC	CCG	TCC	TGA	CCG	TCC	CCC	TTC	GGT	CCG	TAG	ATG	TCT	AAA	CAC

	560					565				570					
Ala	Pro	Gly	Glu	Arg	Pro	Pro	Gly	Met	Phe	Asp	Ser	Ser	Val	Leu	Cys
GCA	CCG	GGG	GAG	CGC	CCT	CCC	GGC	ATG	TTC	GAC	TCG	TCC	GTC	CTC	TGT
CGT	GGC	CCC	CTC	GCG	GGA	GGG	CCG	TAC	AAG	CTG	AGC	AGG	CAG	GAG	ACA

575					580				585					590	
Glu	Cys	Tyr	Asp	Ala	Gly	Cys	Ala	Trp	Tyr	Glu	Leu	Thr	Pro	Ala	Glu
GAG	TGC	TAT	GAC	GCA	GGC	TGT	GCT	TGG	TAT	GAG	CTC	ACG	CCC	GCC	GAG
CTC	ACG	ATA	CTG	CGT	CCG	ACA	CGA	ACC	ATA	CTC	GAG	TGC	GGG	CGG	CTC

				595				600					605		
Thr	Thr	Val	Arg	Leu	Arg	Ala	Tyr	Met	Asn	Thr	Pro	Gly	Leu	Pro	Val
ACT	ACA	GTT	AGG	CTA	CGA	GCG	TAC	ATG	AAC	ACC	CCG	GGG	CTT	CCC	GTG
TGA	TGT	CAA	TCC	GAT	GCT	CGC	ATG	TAC	TTG	TGG	GGC	CCC	GAA	GGG	CAC

Figure 1 (continued)

			610					615				620			
Cys	Gln	Asp	His	Leu	Glu	Phe	Trp	Glu	Gly	Val	Phe	Thr	Gly	Leu	Thr
TGC	CAG	GAC	CAT	CTT	GAA	TTT	TGG	GAG	GGC	GTC	TTT	ACA	GGC	CTC	ACT
ACG	GTC	CTG	GTA	GAA	CTT	AAA	ACC	CTC	CCG	CAG	AAA	TGT	CCG	GAG	TGA

625				630				635							
His	Ile	Asp	Ala	His	Phe	Leu	Ser	Gln	Thr	Lys	Gln	Ser	Gly	Glu	Asn
CAT	ATA	GAT	GCC	CAC	TTT	CTA	TCC	CAG	ACA	AAG	CAG	AGT	GGG	GAG	AAC
GTA	TAT	CTA	CGG	GTG	AAA	GAT	AGG	GTC	TGT	TTC	GTC	TCA	CCC	CTC	TTG

640						645					650				
Leu	Pro	Tyr	Leu	Val	Ala	Tyr	Gln	Ala	Thr	Val	Cys	Ala	Arg	Ala	Gln
CTT	CCT	TAC	CTG	GTA	GCG	TAC	CAA	GCC	ACC	GTG	TGC	GCT	AGG	GCT	CAA
GAA	GGA	ATG	GAC	CAT	CGC	ATG	GTT	CGG	TGG	CAC	ACG	CGA	TCC	CGA	GTT

655					660					665					670				
Ala	Pro	Pro	Pro	Ser	Trp	Asp	Gln	Met	Trp	Lys	Cys	Leu	Ile	Arg	Leu				
GCC	CCT	CCC	CCA	TCG	TGG	GAC	CAG	ATG	TGG	AAG	TGT	TTG	ATT	CGC	CTC				
CGG	GGA	GGG	GGT	AGC	ACC	CTG	GTC	TAC	ACC	TTC	ACA	AAC	TAA	GCG	GAG				

				675					680					685		
Lys	Pro	Thr	Leu	His	Gly	Pro	Thr	Pro	Leu	Leu	Tyr	Arg	Leu	Gly	Ala	
AAG	CCC	ACC	CTC	CAT	GGG	CCA	ACA	CCC	CTG	CTA	TAC	AGA	CTG	GGC	GCT	
TTC	GGG	TGG	GAG	GTA	CCC	GGT	TGT	GGG	GAC	GAT	ATG	TCT	GAC	CCG	CGA	

Figure 1 (continued)

C20c:

Asn Ser Glu Asn Gln Val Glu Gly Glu Val Gln Ile Val Ser Thr Ala
 AAT TCG GAA AAC CAA GTG GAG GGT GAG GTC CAG ATT GTG TCA ACT GCT
 TTA AGC CTT TTG GTT CAC CTC CCA CTC CAG GTC TAA CAC AGT TGA CGA

↑
 EcoRI

Ala Gln Thr Phe Leu Ala Thr Cys Ile Asn Gly Val Cys Trp Thr Val
 GCC CAA ACC TTC CTG GCA ACG TGC ATC AAT GGG GTG TGC TGG ACT GTC
 CGG GTT TGG AAG GAC CGT TGC ACG TAG TTA CCC CAC ACG ACC TGA CAG

↑
 SfaNI

Tyr His Gly Ala Gly Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val
 TAC CAC GGG GCC GGA ACG AGG ACC ATC GCG TCA CCC AAG GGT CCT GTC
 ATG GTG CCC CGG CCT TGC TCC TGG TAG CGC AGT GGG TTC CCA GGA CAG

Ile Gln Met Tyr Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala
 ATC CAG ATG TAT ACC AAT GTA GAC CAA GAC CTT GTG GGC TGG CCC GCT
 TAG GTC TAC ATA TGG TTA CAT CTG GTT CTG GAA CAC CCG ACC GGG CGA

Ser Gln Gly Thr Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp
 TCG CAA GGT ACC CGC TCA TTG ACA CCC TGC ACT TGC GGC TCC TCG GAC
 AGC GTT CCA TGG GCG AGT AAC TGT GGG ACG TGA ACG CCG AGG AGC CTG

Leu Tyr Leu Val Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg
 CTT TAC CTG GTC ACG AGG CAC GCC GAT GTC ATT CCC GTG CGC CGG CGG
 GAA ATG GAC CAG TGC TCC GTG CGG CTA CAG TAA GGG CAC GCG GCC GCC

↑
 NaeI

Gly Asp Ser Arg Gly Ser Leu Val Ser Pro Arg Pro Ile Ser Tyr Leu
 GGT GAT AGC AGG GGC AGC CTC GTG TCG CCC CGG CCC ATT TCC TAC TTG
 CCA CTA TCG TCC CCG TCG GAG CAC AGC GGG GCC GGG TAA AGG ATG AAC

Lys Gly Ser Ser Gly Gly Pro Leu Pro Asn
 AAA GGC TCC TCG GGG GGT CCG CTG CCG AAT TC
 TTT CCG AGG AGC CCC CCA GGC GAC GGC TTA AG

↑
 EcoRI

Figure 2

C26d:

Glu Phe Gly Gly Leu Leu Leu Cys Pro Ala Ala Ala Val Gly Ile Phe
 GAA TTC GGG GGC CTG CTG TTG TGC CCC GCG GCA GCC GTG GGC ATA TTT
 CTT AAG CCC CCG GAC GAC AAC ACG GGG CGC CGT CGG CAC CCG TAT AAA
 ↑
 EcoRI

Arg Ala Ala Val Cys Thr Arg Gly Val Ala Lys Ala Val Asp Phe Ile
 AGG GCC GCG GTG TGC ACC CGT GGA GTG GCT AAG GCG GTG GAC TTT ATC
 TCC CGG CGC CAC ACG TGG GCA CCT CAC CGA TTC CGC CAC CTG AAA TAG
 ↑
 DdeI

Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro Val Phe Thr Asp
 CCT GTG GAG AAC CTA GAG ACA ACC ATG AGG TCC CCG GTG TTC ACG GAT
 GGA CAC CTC TTG GAT CTC TGT TGG TAC TCC AGG GGC CAC AAG TGC CTA

Asn Ser Ser Pro Pro Val Val Pro Gln Ser Phe Gln Val Ala His Leu
 AAC TCC TCT CCA CCA GTA GTG CCC CAG AGC TTC CAG GTG GCT CAC CTC
 TTG AGG AGA GGT GGT CAT CAC GGG GTC TCG AAG GTC CAC CGA GTG GAG
 ↑
 EcoRII

His Ala Pro Arg Ile
 CAT GCT CCC CGA ATT C
 GTA CGA GGG GCT TAA G
 ↑
 EcoRI

Figure 3

C8h:

Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr Arg His Ala
 CCC TGC ACT TGC GGC TCC TCG GAC CTT TAC CTG GTC ACG AGG CAC GCC
 GGG ACG TGA ACG CCG AGG AGC CTG GAA ATG GAC CAG TGC TCC GTG CGG

Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg Gly Ser Leu Leu
 GAT GTC ATT CCC GTG CGC CGG CGG GGT GAT AGC AGG GGC AGC CTG CTG
 CTA CAG TAA GGG CAC GCG GCC GCC CCA CTA TCG TCC CCG TCG GAC GAC

Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser Ser Gly Gly Pro Leu
 TCG CCC CGG CCC ATT TCC TAC TTG AAA GGC TCC TCG GGG GGT CCG CTG
 AGC GGG GCC GGG TAA AGG ATG AAC TTT CCG AGG AGC CCC CCA GGC GAC

Leu Cys Pro Ala Gly His Ala Val Gly Ile Phe Arg Ala Ala Val Cys
 TTG TGC CCC GCG GGG CAC GCC GTG GGC ATA TTT AGG GCC GCG GTG TGC
 AAC ACG GGG CGC CCC GTG CGG CAC CCG TAT AAA TCC CGG CGC CAC ACG

Thr Arg Gly Val Ala Lys Ala Val Asp Phe Ile Pro Val Glu Asn Leu
 ACC CGT GGA GTG GCT AAG GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA
 TGG GCA CCT CAC CGA TTC CGC CAC CTG AAA TAG GGA CAC CTC TTG GAT

↑
 DdeI

Glu Thr Thr Met Arg Ser Pro Val Phe Thr Asp Asn Ser
 GAG ACA ACC ATG AGG TCC CCG GTG TTC ACG GAT AAC TCC TC
 CTC TGT TGG TAC TCC AGG GGC CAC AAG TGC CTA TTG AGG AG

Figure 4

C7f:

Ile Arg Gly Thr Tyr Val Tyr Asn His Leu Thr Pro Leu Arg Asp Trp
 ATT CGG GGC ACC TAT GTT TAT AAC CAT CTC ACT CCT CTT CGG GAC TGG
 TAA GCC CCG TGG ATA CAA ATA TTG GTA GAG TGA GGA GAA GCC CTG ACC

↑
 EcoRI

Ala His Asn Gly Leu Arg Asp Leu Ala Val Ala Val Glu Pro Val Val
 GCG CAC AAC GGC TTG CGA GAT CTG GCC GTG GCT GTA GAG CCA GTC GTC
 CGC GTG TTG CCG AAC GCT CTA GAC CGG CAC CGA CAT CTC GGT CAG CAG

Phe Ser Gln Met Glu Thr Lys Leu Ile Thr Trp Gly Ala Asp Thr Ala
 TTC TCC CAA ATG GAG ACC AAG CTC ATC ACG TGG GGG GCA GAT ACC GCC
 AAG AGG GTT TAC CTC TGG TTC GAG TAG TGC ACC CCC CGT CTA TGG CCG

Ala Cys Gly Asp Ile Ile Asn Gly Leu Pro Val Ser Ala Arg Arg Gly
 GCG TGC GGT GAC ATC ATC AAC GGC TTG CCT GTT TCC GCC CGC AGG GGC
 CGC ACG CCA CTG TAG TAG TTG CCG AAC GGA CAA AGG CGG GCG TCC CCG

Arg Glu Ile Leu Leu Gly Pro Ala Asp Gly Met Val Ser Lys Gly Trp
 CGG GAG ATA CTG CTC GGG CCA GCC GAT GGA ATG GTC TCC AAG GGT TGG
 GCC CTC TAT GAC GAG CCC GGT CGG CTA CCT TAC CAG AGG TTC CCA ACC

Arg Leu Leu Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu
 AGG TTG CTG GCG CCC ATC ACG GCG TAC GCC CAG CAG ACA AGG GGC CTC
 TCC AAC GAC CGC GGG TAG TGC CGC ATG CGG GTC GTC TGT TCC CCG GAG

Leu Gly Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val
 CTA GGG TGC ATA ATC ACC AGC CTA ACT GGC CGG GAC AAA AAC CAA GTG
 GAT CCC ACG TAT TAG TGG TCG GAT TGA CCG GCC CTG TTT TTG GTT CAC

Glu Gly Glu Val Gln Ile Val Ser Thr Ala Ala Gln Thr Phe Leu Ala
 GAG GGT GAG GTC CAG ATT GTG TCA ACT GCT GCC CAA ACC TTC CTG GCA
 CTC CCA CTC CAG GTC TAA CAC AGT TGA CGA CGG GTT TGG AAG GAC CGT

Thr Cys Ile Asn Gly Val Cys Trp Pro Asn
 ACG TGC ATC AAT GGG GTG TGC TGG CCG AAT TC
 TGC ACG TAG TTA CCC CAC ACG ACC GGC TTA AG

↑
 SfaNI

↑
 EcoRI

Figure 5

C31:

Glu Phe Gly Ser Val Ile Pro Thr Ser Gly Asp Val Val Val Val Ala
 GAA TTC GGG TCC GTC ATC CCG ACC AGC GGC GAT GTT GTC GTC GTC GCA
 CTT AAG CCC AGG CAG TAG GGC TGG TCG CCG CTA CAA CAG CAG CAG CGT

↑
 EcoRI

Thr Asp Ala Leu Met Thr Gly Tyr Thr Gly Asp Phe Asp Ser Val Ile
 ACC GAT GCC CTC ATG ACC GGC TAT ACC GGC GAC TTC GAC TCG GTG ATA
 TGG CTA CGG GAG TAC TGG CCG ATA TGG CCG CTG AAG CTG AGC CAC TAT

↑
 HinfI

Asp Cys Asn Thr Cys Val Thr Gln Thr Val Asp Phe Ser Leu Asp Pro
 GAC TGC AAT ACG TGT GTC ACC CAG ACA GTC GAT TTC AGC CTT GAC CCT
 CTG ACG TTA TGC ACA CAG TGG GTC TGT CAG CTA AAG TCG GAA CTG GGA

Thr Phe Thr Ile Glu Thr Ile Thr Leu Pro Gln Asp Ala Val Ser Arg
 ACC TTC ACC ATT GAG ACA ATC ACG CTC CCC CAA GAT GCT GTC TCC CGC
 TGG AAG TGG TAA CTC TGT TAG TGC GAG GGG GTT CTA CGA CAG AGG GCG

Thr Gln Arg Arg Gly Arg Thr Gly Arg Gly Lys Pro Gly Ile Tyr Arg
 ACT CAA CGT CGG GGC AGG ACT GGC AGG GGG AAG CCA GGC ATC TAC AGA
 TGA GTT GCA GCC CCG TCC TGA CCG TCC CCC TTC GGT CCG TAG ATG TCT

Phe Val Ala Pro Gly Glu Arg Pro Ser Gly Met Phe Asp Ser Ser Val
 TTT GTG GCA CCG GGG GAG CGC CCC TCC GGC ATG TTC GAC TCG TCC GTC
 AAA CAC CGT GGC CCC CTC GCG GGG AGG CCG TAC AAG CTG AGC AGG CAG

↑
 BglI

↑
 HinfI

Leu Cys Glu Cys Pro Asn
 CTC TGT GAG TGC CCG AAT TC
 GAG ACA CTC ACG GGC TTA AG

↑
 EcoRI

Figure 6

C35:

Ile Arg Ser Ile Glu Thr Ile Thr Leu Pro Gln Asp Ala Val Ser Arg
 ATT CGG TCC ATT GAG ACA ATC ACG CTC CCC CAG GAT GCT GTC TCC CGC
 TAA GCC AGG TAA CTC TGT TAG TGC GAG GGG GTC CTA CGA CAG AGG GCG

↑

EcoRI

Thr Gln Arg Arg Gly Arg Thr Gly Arg Gly Lys Pro Gly Ile Tyr Arg
 ACT CAA CGT CGG GGC AGG ACT GGC AGG GGG AAG CCA GGC ATC TAC AGA
 TGA GTT GCA GCC CCG TCC TGA CCG TCC CCC TTC GGT CCG TAG ATG TCT

Phe Val Ala Pro Gly Glu Arg Pro Ser Gly Met Phe Asp Ser Ser Val
 TTT GTG GCA CCG GGG GAG CGC CCC TCC GGC ATG TTC GAC TCG TCC GTC
 AAA CAC CGT GGC CCC CTC GCG GGG AGG CCG TAC AAG CTG AGC AGG CAG

↑

BglI

Leu Cys Glu Cys Tyr Asp Ala Gly Cys Ala Trp Tyr Glu Leu Thr Pro
 CTC TGT GAG TGC TAT GAC GCA GGC TGT GCT TGG TAT GAG CTC ACG CCC
 GAG ACA CTC ACG ATA CTG CGT CCG ACA CGA ACC ATA CTC GAG TGC GGG

Ala Glu Thr Thr Val Arg Leu Arg Ala Tyr Met Asn Thr Pro Gly Leu
 GCC GAG ACT ACA GTT AGG CTA CGA GCG TAC ATG AAC ACC CCG GGG CTT
 CGG CTC TGA TGT CAA TCC GAT GCT CGC ATG TAC TTG TGG GGC CCC GAA

Pro Val Cys Gln Asp His Leu Glu Phe Trp Glu Gly Val Phe Thr Gly
 CCC GTG TGC CAG GAC CAT CTT GAA TTT TGG GAG GGC GTC TTT ACA GGC
 GGG CAC ACG GTC CTG GTA GAA CTT AAA ACC CTC CCG CAG AAA TGT CCG

Leu Thr His Ile Asp Ala His Phe Leu Ser Gln Thr Lys Gln Ser Gly
 CTC ACT CAT ATA GAT GCC CAC TTT CTA TCC CAG ACA AAG CAG AGT GGG
 GAG TGA GTA TAT CTA CGG GTG AAA GAT AGG GTC TGT TTC GTC TCA CCC

Glu Asn Leu Pro Tyr Leu Val Ala Tyr Gln Ala Thr Val Cys Ala Arg
 GAG AAC CTT CCT TAC CTG GTA GCG TAC CAA GCC ACC GTG TGC GCT AGG
 CTC TTG GAA GGA ATG GAC CAT CGC ATG GTT CCG TGG CAC ACG CGA TCC

Ala Gln Ala Pro Pro Pro Ser Trp Asp Gln Met Trp Lys Cys Leu Ile
 GCT CAA GCC CCT CCC CCA TCG TGG GAC CAG ATG TGG AAG TGT TTG ATT
 CGA GTT CGG GGA GGG GGT AGC ACC CTG GTC TAC ACC TTC ACA AAC TAA

Arg Leu Lys Pro Thr Leu His Gly Pro Thr Pro Leu Leu Tyr Arg Leu
 CGC CTC AAG CCC ACC CTC CAT GGG CCA ACA CCC CTG CTA TAC AGA CTG
 GCG GAG TTC GGG TGG GAG GTA CCC GGT TGT GGG GAC GAT ATG TCT GAC

Gly Ala Ala Glu Phe
 GGC GCT GCC GAA TTC
 CCG CGA CGG CTT AAG

↑

EcoRI

Figure 7

C33c:

Glu Phe Gly Ala Val Asp Phe Ile Pro Val Glu Asn Leu Glu Thr Thr
 GAA TTC GGG GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC
 CTT AAG CCC CGC CAC CTG AAA TAG GGA CAC CTC TTG GAT CTC TGT TGG

↑
 EcoRI

Met Arg Ser Pro Val Phe Thr Asp Asn Ser Ser Pro Pro Val Val Pro
 ATG AGG TCC CCG GTG TTC ACG GAT AAC TCC TCT CCA CCA GTA GTG CCC
 TAC TCC AGG GGC CAC AAG TGC CTA TTG AGG AGA GGT GGT CAT CAC GGG

Gln Ser Phe Gln Val Ala His Leu His Ala Pro Thr Gly Ser Gly Lys
 CAG AGC TTC CAG GTG GCT CAC CTC CAT GCT CCC ACA GGC AGC GGC AAA
 GTC TCG AAG GTC CAC CGA GTG GAG GTA CGA GGG TGT CCG TCG CCG TTT

Ser Thr Lys Val Pro Ala Ala Tyr Ala Ala Gln Gly Tyr Lys Val Leu
 AGC ACC AAG GTC CCG GCT GCA TAT GCA GCT CAG GGC TAT AAG GTG CTA
 TCG TGG TTC CAG GGC CGA CGT ATA CGT CGA GTC CCG ATA TTC CAC GAT

Val Leu Asn Pro Ser Val Ala Ala Thr Leu Gly Phe Gly Ala Tyr Met
 GTA CTC AAC CCC TCT GTT GCT GCA ACA CTG GGC TTT GGT GCT TAC ATG
 CAT GAG TTG GGG AGA CAA CGA CGT TGT GAC CCG AAA CCA CGA ATG TAC

Ser Lys Ala His Gly Ile Asp Pro Asn Ile Arg Thr Gly Val Arg Thr
 TCC AAG GCT CAT GGG ATC GAT CCT AAC ATC AGG ACC GGG GTG AGA ACA
 AGG TTC CGA GTA CCC TAG CTA GGA TTG TAG TCC TGG CCC CAC TCT TGT

Ile Thr Thr Gly Ser Pro Ile Thr Tyr Ser Thr Tyr Gly Lys Phe Leu
 ATT ACC ACT GGC AGC CCC ATC ACG TAC TCC ACC TAC GGC AAG TTC CTT
 TAA TGG TGA CCG TCG GGG TAG TGC ATG AGG TGG ATG CCG TTC AAG GAA

Ala Asp Gly Gly Cys Ser Gly Gly Ala Tyr Asp Ile Ile Ile Cys Asp
 GCC GAC GGC GGG TGC TCG GGG GGC GCT TAT GAC ATA ATA ATT TGT GAC
 CGG CTG CCG CCC ACG AGC CCC CCG CGA ATA CTG TAT TAT TAA ACA CTG

Glu Cys His Ser Thr Asp Ala Thr Ser Ile Leu Gly Ile Gly Thr Val
 GAG TGC CAC TCC ACG GAT GCC ACA TCC ATC TTG GGC ATT GGC ACT GTC
 CTC ACG GTG AGG TGC CTA CGG TGT AGG TAG AAC CCG TAA CCG TGA CAG

Figure 8

Leu Asp Gln Ala Glu Thr Ala Gly Ala Arg Leu Val Val Leu Ala Thr
 CTT GAC CAA GCA GAG ACT GCG GGG GCG AGA CTG GTT GTG CTC GCC ACC
 GAA CTG GTT CGT CTC TGA CGC CCC CGC TCT GAC CAA CAC GAG CGG TGG

Ala Thr Pro Pro Gly Ser Val Thr Val Pro His Pro Asn Ile Glu Glu
 GCC ACC CCT CCG GGC TCC GTC ACT GTG CCC CAT CCC AAC ATC GAG GAG
 CGG TGG GGA GGC CCG AGG CAG TGA CAC GGG GTA GGG TTG TAG CTC CTC

Val Ala Leu Ser Thr Thr Gly Glu Ile Pro Phe Tyr Gly Lys Ala Ile
 GTT GCT CTG TCC ACC ACC GGA GAG ATC CCT TTT TAC GGC AAG GCT ATC
 CAA CGA GAC AGG TGG TGG CCT CTC TAG GGA AAA ATG CCG TTC CGA TAG

Pro Leu Glu Val Ile Lys Gly Gly Arg His Leu Ile Phe Cys His Ser
 CCC CTC GAA GTA ATC AAG GGG GGG AGA CAT CTC ATC TTC TGT CAT TCA
 GGG GAG CTT CAT TAG TTC CCC CCC TCT GTA GAG TAG AAG ACA GTA AGT

Lys Lys Lys Cys Asp Glu Leu Ala Ala Lys Leu Val Ala Leu Gly Ile
 AAG AAG AAG TGC GAC GAA CTC GCC GCA AAG CTG GTC GCA TTG GGC ATC
 TTC TTC TTC ACG CTG CTT GAG CGG CGT TTC GAC CAG CGT AAC CCG TAG

Asn Ala Val Ala Tyr Tyr Arg Gly Leu Asp Val Ser Val Ile Pro Thr
 AAT GCC GTG GCC TAC TAC CGC GGT CTT GAC GTG TCC GTC ATC CCG ACC
 TTA CGG CAC CGG ATG ATG GCG CCA GAA CTG CAC AGG CAG TAG GGC TGG

Ser Gly Asp Val Val Val Val Ala Thr Asp Ala Leu Met Thr Gly Tyr
 AGC GGC GAT GTT GTC GTC GTG GCA ACC GAT GCC CTC ATG ACC GGC TAT
 TCG CCG CTA CAA CAG CAG CAC CGT TGG CTA CGG GAG TAC TGG CCG ATA

Thr Gly Asp Phe Asp Ser Val Ile Asp Cys Asn Thr Cys Ala Glu Phe
 ACC GGC GAC TTC GAC TCG GTG ATA GAC TGC AAT ACG TGT GCC GAA TTC
 TGG CCG CTG AAG CTG AGC CAC TAT CTG ACG TTA TGC ACA CGG CTT AAG

↑
 HinfI

↑
 EcoRI

Figure 8 (Continued)

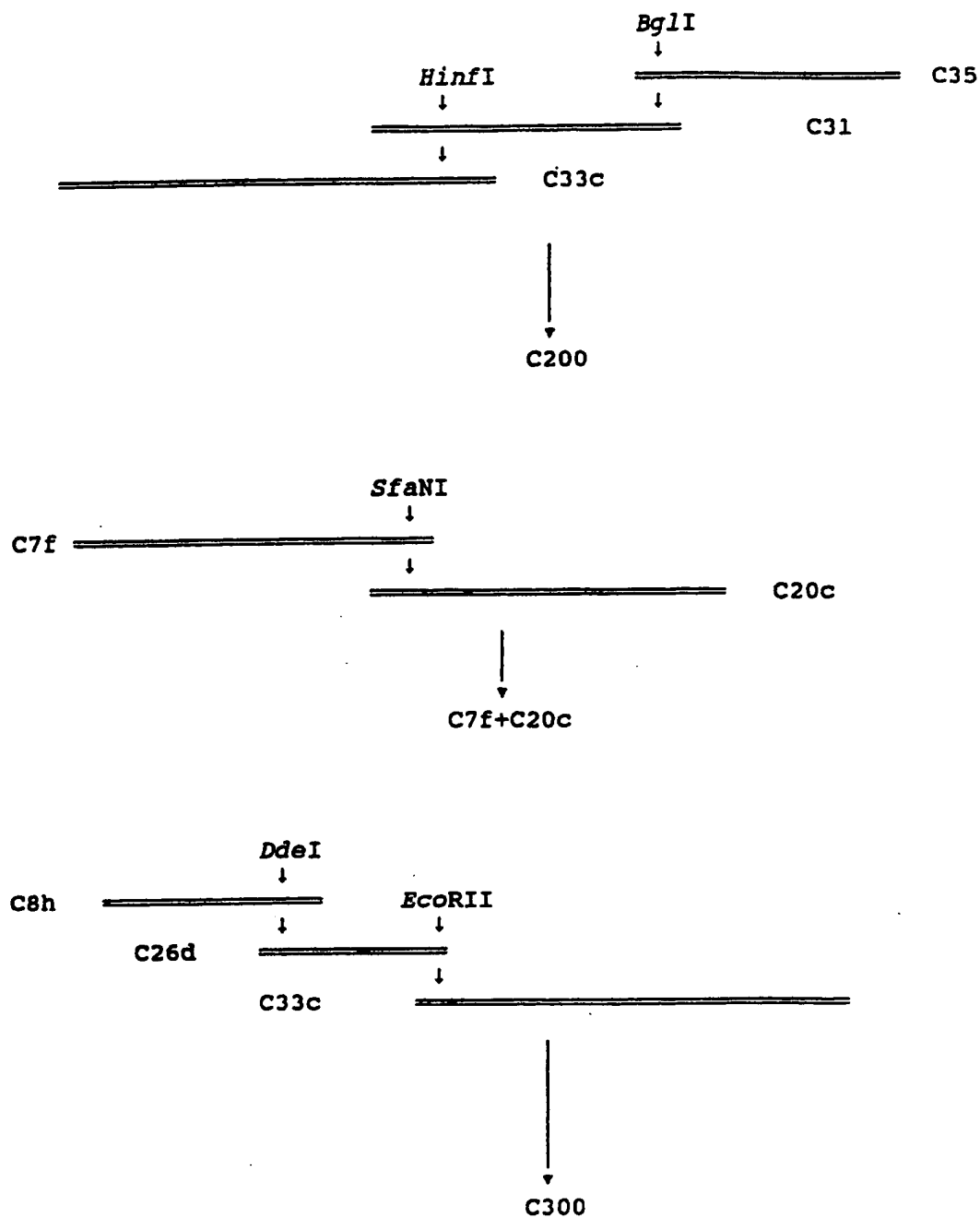


Figure 9

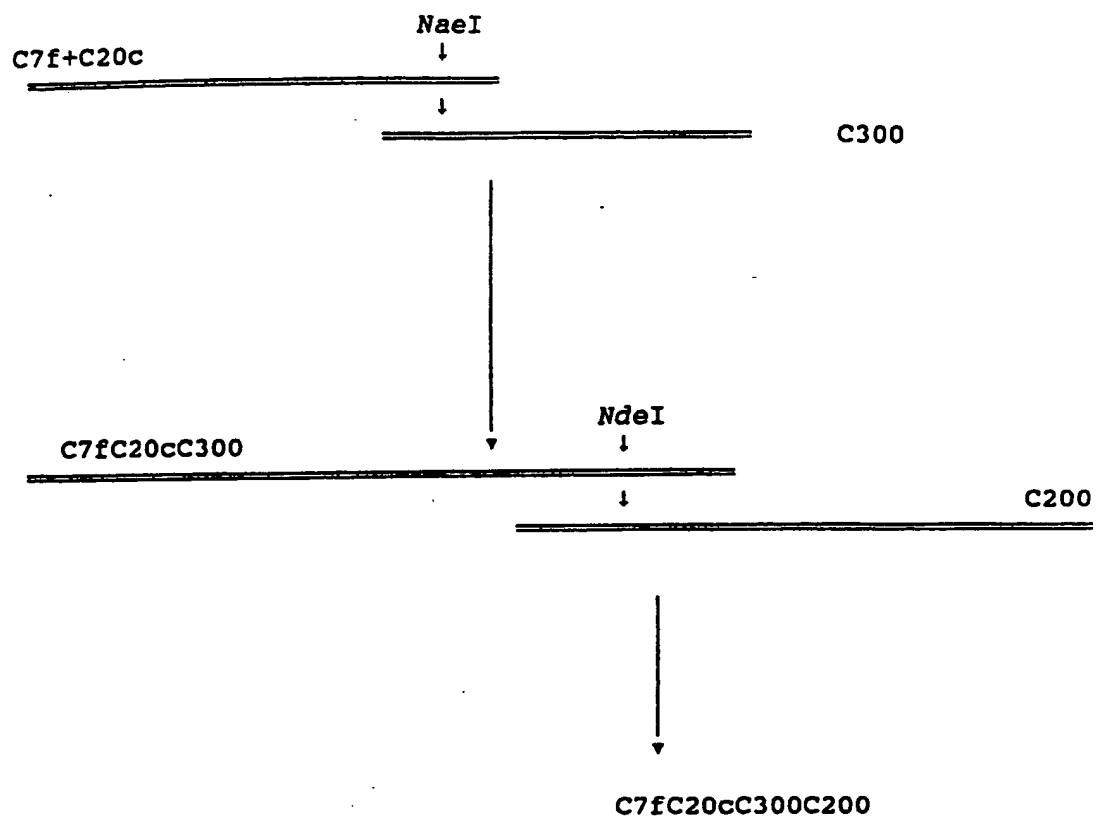


Figure 9 (Continued)

-155 -150
 Met Ala Thr Asn Pro Val Cys Val Leu
 ATG GCT ACA AAC CCT GTT TGC GTT TTG
 TAC CGA TGT TTG GGA CAA ACG CAA AAC

-145 -140 -135
 Lys Gly Asp Gly Pro Val Gln Gly Ile Ile Asn Phe Glu Gln Lys Glu
 AAG GGT GAC GGC CCA GTT CAA GGT ATT ATT AAC TTC GAG CAG AAG GAA
 TTC CCA CTG CCG GGT CAA GTT CCA TAA TAA TTG AAG CTC GTC TTC CTT

-130 -125 -120 -115
 Ser Asn Gly Pro Val Lys Val Trp Gly Ser Ile Lys Gly Leu Thr Glu
 AGT AAT GGA CCA GTG AAG GTG TGG GGA AGC ATT AAA GGA CTG ACT GAA
 TCA TTA CCT GGT CAC TTC CAC ACC CCT TCG TAA TTT CCT GAC TGA CTT

-110 -105 -100
 Gly Leu His Gly Phe His Val His Glu Phe Gly Asp Asn Thr Ala Gly
 GGC CTG CAT GGA TTC CAT GTT CAT GAG TTT GGA GAT AAT ACA GCA GGC
 CCG GAC GTA CCT AAG GTA CAA GTA CTC AAA CCT CTA TTA TGT CGT CCG

-95 -90 -85
 Cys Thr Ser Pro Gly Pro His Phe Asn Pro Leu Ser Arg Lys His Gly
 TGT ACC AGT CCA GGT CCT CAC TTT AAT CCT CTA TCC AGA AAA CAC GGT
 ACA TGG TCA GGT CCA GGA GTG AAA TTA GGA GAT AGG TCT TTT GTG CCA

-80 -75 -70
 Gly Pro Lys Asp Glu Glu Arg His Val Gly Asp Leu Gly Asn Val Thr
 GGG CCA AAG GAT GAA GAG AGG CAT GTT GGA GAC TTG GGC AAT GTG ACT
 CCC GGT TTC CTA CTT CTC TCC GTA CAA CCT CTG AAC CCG TTA CAC TGA

-65 -60 -55
 Ala Asp Lys Asp Gly Val Ala Asp Val Ser Ile Glu Asp Ser Val Ile
 GCT GAC AAA GAT GGT GTG GCC GAT GTG TCT ATT GAA GAT TCT GTG ATC
 CGA CTG TTT CTA CCA CAC CGG CTA CAC AGA TAA CTT CTA AGA CAC TAG

-50 -45 -40 -35
 Ser Leu Ser Gly Asp His Cys Ile Ile Gly Arg Thr Leu Val Val His
 TCA CTC TCA GGA GAC CAT TGC ATC ATT GGC CGC ACA CTG GTG GTC CAT
 AGT GAG AGT CCT CTG GTA ACG TAG TAA CCG GCG TGT GAC CAC CAG GTA

-30 -25 -20
 Glu Lys Ala Asp Asp Leu Gly Lys Gly Gly Asn Glu Glu Ser Thr Lys
 GAA AAA GCA GAT GAC TTG GGC AAA GGT GGA AAT GAA GAA AGT ACA AAG
 CTT TTT CGT CTA CTG AAC CCG TTT CCA CCT TTA CTT CTT TCA TGT TTC

-15 -10 -5
 Thr Gly Asn Ala Gly Ser Arg Leu Ala Cys Gly Val Ile Gly Ile Arg
 ACA GGA AAC GCT GGA AGT CGT TTG GCT TGT GGT GTA ATT GGG ATC CGA
 TGT CCT TTG CGA CCT TCA GCA AAC CGA ACA CCA CAT TAA CCC TAG GCT

Figure 10

Figure 10 (continued)

253					260					265					270
Glu	Asn	Leu	Glu	Thr	Thr	Met	Arg	Ser	Pro	Val	Phe	Thr	Asp	Asn	Ser
GAG	AAC	CTA	GAG	ACA	ACC	ATG	AGG	TCC	CCG	GTG	TTC	ACG	GAT	AAC	TCC
CTC	TTG	GAT	CTC	TGT	TGG	TAC	TCC	AGG	GGC	CAC	AAG	TGC	CTA	TTG	AGG
				275						280					285
Ser	Pro	Pro	Val	Val	Pro	Gln	Ser	Phe	Gln	Val	Ala	His	Leu	His	Ala
TCT	CCA	CCA	GTA	GTG	CCC	CAG	AGC	TTC	CAG	GTG	GCT	CAC	CTC	CAT	GCT
AGA	GGT	GGT	CAT	CAC	GGG	GTC	TCG	AAG	GTC	CAC	CGA	GTG	GAG	GTA	CGA
			290						295					300	
Pro	Thr	Gly	Ser	Gly	Lys	Ser	Thr	Lys	Val	Pro	Ala	Ala	Tyr	Ala	Ala
CCC	ACA	GGC	AGC	GGC	AAA	AGC	ACC	AAG	GTC	CCG	GCT	GCA	TAT	GCA	GCT
GGG	TGT	CCG	TCG	CCG	TTT	TCG	TGG	TTC	CAG	GGC	CGA	CGT	ATA	CGT	CGA
↑															
NdeI															
		305						310				315			
Gln	Gly	Tyr	Lys	Val	Leu	Val	Leu	Asn	Pro	Ser	Val	Ala	Ala	Thr	Leu
CAG	GGC	TAT	AAG	GTG	CTA	GTA	CTC	AAC	CCC	TCT	GTT	GCT	GCA	ACA	CTG
GTC	CCG	ATA	TTC	CAC	GAT	CAT	GAG	TTG	GGG	AGA	CAA	CGA	CGT	TGT	GAC
		320				325				330					
Gly	Phe	Gly	Ala	Tyr	Met	Ser	Lys	Ala	His	Gly	Ile	Asp	Pro	Asn	Ile
GGC	TTT	GGT	GCT	TAC	ATG	TCC	AAG	GCT	CAT	GGG	ATC	GAT	CCT	AAC	ATC
CCG	AAA	CCA	CGA	ATG	TAC	AGG	TTC	CGA	GTA	CCC	TAG	CTA	GGA	TTG	TAG
		335			340					345					350
Arg	Thr	Gly	Val	Arg	Thr	Ile	Thr	Thr	Gly	Ser	Pro	Ile	Thr	Tyr	Ser
AGG	ACC	GGG	GTG	AGA	ACA	ATT	ACC	ACT	GGC	AGC	CCC	ATC	ACG	TAC	TCC
TCC	TGG	CCC	CAC	TCT	TGT	TAA	TGG	TGA	CCG	TCG	GGG	TAG	TGC	ATG	AGG
			355						360				365		
Thr	Tyr	Gly	Lys	Phe	Leu	Ala	Asp	Gly	Gly	Cys	Ser	Gly	Gly	Ala	Tyr
ACC	TAC	GGC	AAG	TTC	CTT	GCC	GAC	GGC	GGG	TGC	TCG	GGG	GGC	GCT	TAT
TGG	ATG	CCG	TTC	AAG	GAA	CGG	CTG	CCG	CCC	ACG	AGC	CCC	CCG	CGA	ATA
		370						375				380			
Asp	Ile	Ile	Ile	Cys	Asp	Glu	Cys	His	Ser	Thr	Asp	Ala	Thr	Ser	Ile
GAC	ATA	ATA	ATT	TGT	GAC	GAG	TGC	CAC	TCC	ACG	GAT	GCC	ACA	TCC	ATC
CTG	TAT	TAT	TAA	ACA	CTG	CTC	ACG	GTG	AGG	TGC	CTA	CGG	TGT	AGG	TAG

Figure 10 (continued)

			385					390					395				
Leu	Gly	Ile	Gly	Thr	Val	Leu	Asp	Gln	Ala	Glu	Thr	Ala	Gly	Ala	Arg		
TTG	GGC	ATT	GGC	ACT	GTC	CTT	GAC	CAA	GCA	GAG	ACT	GCG	GGG	GCG	AGA		
AAC	CCG	TAA	CCG	TGA	CAG	GAA	CTG	GTT	CGT	CTC	TGA	CGC	CCC	CGC	TCT		
		400					405					410					
Leu	Val	Val	Leu	Ala	Thr	Ala	Thr	Pro	Pro	Gly	Ser	Val	Thr	Val	Pro		
CTG	GTT	GTG	CTC	GCC	ACC	GCC	ACC	CCT	CCG	GGC	TCC	GTC	ACT	GTG	CCC		
GAC	CAA	CAC	GAG	CGG	TGG	CGG	TGG	GGA	GGC	CCG	AGG	CAG	TGA	CAC	GGG		
415						420					425					430	
His	Pro	Asn	Ile	Glu	Glu	Val	Ala	Leu	Ser	Thr	Thr	Gly	Glu	Ile	Pro		
CAT	CCC	AAC	ATC	GAG	GAG	GTT	GCT	CTG	TCC	ACC	ACC	GGA	GAG	ATC	CCT		
GTA	GGG	TTG	TAG	CTC	CTC	CAA	CGA	GAC	AGG	TGG	TGG	CCT	CTC	TAG	GGA		
			435							440					445		
Phe	Tyr	Gly	Lys	Ala	Ile	Pro	Leu	Glu	Val	Ile	Lys	Gly	Gly	Arg	His		
TTT	TAC	GGC	AAG	GCT	ATC	CCC	CTC	GAA	GTA	ATC	AAG	GGG	GGG	AGA	CAT		
AAA	ATG	CCG	TTC	CGA	TAG	GGG	GAG	CTT	CAT	TAG	TTC	CCC	CCC	TCT	GTA		
			450					455						460			
Leu	Ile	Phe	Cys	His	Ser	Lys	Lys	Lys	Cys	Asp	Glu	Leu	Ala	Ala	Lys		
CTC	ATC	TTC	TGT	CAT	TCA	AAG	AAG	AAG	TGC	GAC	GAA	CTC	GCC	GCA	AAG		
GAG	TAG	AAG	ACA	GTA	AGT	TTC	TTC	TTC	ACG	CTG	CTT	GAG	CGG	CGT	TTC		
		465					470						475				
Leu	Val	Ala	Leu	Gly	Ile	Asn	Ala	Val	Ala	Tyr	Tyr	Arg	Gly	Leu	Asp		
CTG	GTC	GCA	TTG	GGC	ATC	AAT	GCC	GTG	GCC	TAC	TAC	CGC	GGT	CTT	GAC		
GAC	CAG	CGT	AAC	CCG	TAG	TTA	CGG	CAC	CGG	ATG	ATG	GCG	CCA	GAA	CTG		
		480				485						490					
Val	Ser	Val	Ile	Pro	Thr	Ser	Gly	Asp	Val	Val	Val	Val	Ala	Thr	Asp		
GTG	TCC	GTC	ATC	CCG	ACC	AGC	GGC	GAT	GTT	GTC	GTC	GTG	GCA	ACC	GAT		
CAC	AGG	CAG	TAG	GGC	TGG	TCG	CCG	CTA	CAA	CAG	CAG	CAC	CGT	TGG	CTA		

Figure 10 (continued)

495		500		505		510
Ala Leu Met Thr Gly Tyr Thr Gly Asp Phe Asp Ser Val Ile Asp Cys						
GCC CTC ATG ACC GGC TAT ACC GGC GAC TTC GAC TCG GTG ATA GAC TGC						
CGG GAG TAC TGG CCG ATA TGG CCG CTG AAG CTG AGC CAC TAT CTG ACG						
	515		520		525	
Asn Thr Cys Val Thr Gln Thr Val Asp Phe Ser Leu Asp Pro Thr Phe						
AAT ACG TGT GTC ACC CAG ACA GTC GAT TTC AGC CTT GAC CCT ACC TTC						
TTA TGC ACA CAG TGG GTC TGT CAG CTA AAG TCG GAA CTG GGA TGG AAG						
	530		535		540	
Thr Ile Glu Thr Ile Thr Leu Pro Gln Asp Ala Val Ser Arg Thr Gln						
ACC ATT GAG ACA ATC ACG CTC CCC CAA GAT GCT GTC TCC CGC ACT CAA						
TGG TAA CTC TGT TAG TGC GAG GGG GTT CTA CGA CAG AGG GCG TGA GTT						
	545		550		555	
Arg Arg Gly Arg Thr Gly Arg Gly Lys Pro Gly Ile Tyr Arg Phe Val						
CGT CGG GGC AGG ACT GGC AGG GGG AAG CCA GGC ATC TAC AGA TTT GTG						
GCA GCC CCG TCC TGA CCG TCC CCC TTC GGT CCG TAG ATG TCT AAA CAC						
	560		565		570	
Ala Pro Gly Glu Arg Pro Pro Gly Met Phe Asp Ser Ser Val Leu Cys						
GCA CCG GGG GAG CGC CCT CCC GGC ATG TTC GAC TCG TCC GTC CTC TGT						
CGT GGC CCC CTC GCG GGA GGG CCG TAC AAG CTG AGC AGG CAG GAG ACA						
	575		580		585	590
Glu Cys Tyr Asp Ala Gly Cys Ala Trp Tyr Glu Leu Thr Pro Ala Glu						
GAG TGC TAT GAC GCA GGC TGT GCT TGG TAT GAG CTC ACG CCC GCC GAG						
CTC ACG ATA CTG CGT CCG ACA CGA ACC ATA CTC GAG TGC GGG CCG CTC						
	595		600		605	
Thr Thr Val Arg Leu Arg Ala Tyr Met Asn Thr Pro Gly Leu Pro Val						
ACT ACA GTT AGG CTA CGA GCG TAC ATG AAC ACC CCG GGG CTT CCC GTG						
TGA TGT CAA TCC GAT GCT CGC ATG TAC TTG TGG GGC CCC GAA GGG CAC						

Figure 10 (continued)

			610					615					620				
Cys	Gln	Asp	His	Leu	Glu	Phe	Trp	Glu	Gly	Val	Phe	Thr	Gly	Leu	Thr		
TGC	CAG	GAC	CAT	CTT	GAA	TTT	TGG	GAG	GGC	GTC	TTT	ACA	GGC	CTC	ACT		
ACG	GTC	CTG	GTA	GAA	CTT	AAA	ACC	CTC	CCG	CAG	AAA	TGT	CCG	GAG	TGA		

		625					630					635					
His	Ile	Asp	Ala	His	Phe	Leu	Ser	Gln	Thr	Lys	Gln	Ser	Gly	Glu	Asn		
CAT	ATA	GAT	GCC	CAC	TTT	CTA	TCC	CAG	ACA	AAG	CAG	AGT	GGG	GAG	AAC		
GTA	TAT	CTA	CGG	GTG	AAA	GAT	AGG	GTC	TGT	TTC	GTC	TCA	CCC	CTC	TTG		

	640					645					650						
Leu	Pro	Tyr	Leu	Val	Ala	Tyr	Gln	Ala	Thr	Val	Cys	Ala	Arg	Ala	Gln		
CTT	CCT	TAC	CTG	GTA	GCG	TAC	CAA	GCC	ACC	GTG	TGC	GCT	AGG	GCT	CAA		
GAA	GGA	ATG	GAC	CAT	CGC	ATG	GTT	CGG	TGG	CAC	ACG	CGA	TCC	CGA	GTT		

655					660					665					670		
Ala	Pro	Pro	Pro	Ser	Trp	Asp	Gln	Met	Trp	Lys	Cys	Leu	Ile	Arg	Leu		
GCC	CCT	CCC	CCA	TCG	TGG	GAC	CAG	ATG	TGG	AAG	TGT	TTG	ATT	CGC	CTC		
CGG	GGA	GGG	GGT	AGC	ACC	CTG	GTC	TAC	ACC	TTC	ACA	AAC	TAA	GCG	GAG		

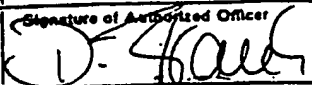
				675					680					685			
Lys	Pro	Thr	Leu	His	Gly	Pro	Thr	Pro	Leu	Leu	Tyr	Arg	Leu	Gly	Ala		
AAG	CCC	ACC	CTC	CAT	GGG	CCA	ACA	CCC	CTG	CTA	TAC	AGA	CTG	GGC	GCT		
TTC	GGG	TGG	GAG	GTA	CCC	GGT	TGT	GGG	GAC	GAT	ATG	TCT	GAC	CCG	CGA		

Figure 10 (continued)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/02209

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁴		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : C 12 Q 1/18, 1/37, 1/70, G 01 N 33/573, 33/576, //C 07 K 13/00, C 12 N 15/51, 15/57, 9/99		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁵	C 12 Q, G 01 N 33/00	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁶		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁴		
Category ⁵	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	CRC Critical Reviews in Biotechnology, vol. 8, issue 2, 1988, B.D. Korant et al. "Viral Proteases: An Emerging Therapeutic Target", pages 149-157, see pages 153, table 4 - page 154, line 41.	1
A	Science, vol. 247, January 26, 1990 (26.01.90), T.J. McQuade et al. "A synthetic HIV-1 Protease inhibitor with antiviral activity arrests HIV-like particle maturation", pages 454-456, see abstract.	1
P, A	EP, A2, 0 414 475 (CHIRON CORPORATION) 27 February 1991 (27.02.91), see page 15, lines 4-17 and	1
<p>¹⁴ Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
11 July 1991	09.08.91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 Mme Dagmar FRANK	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,A	<p>claim 17.</p> <p>EP, A1, 0 388 232 (CHIRON CORPORATION) 19 September 1990 (19.09.90), see page 22, line 19 - page 23, line 3.</p>	1

ANHANG
zum internationalen Recherchen-
bericht über die internationale
Patentanmeldung Nr.

ANNEX
to the International Search
Report to the International Patent
Application No.

ANNEXE
au rapport de recherche inter-
national relatif à la demande de brevet
international n°

PCT/US 91/02209 SAE 46369

In diesem Anhang sind die Mitglieder
der Patentfamilien der in obenge-
nannten internationalen Recherchenbericht
angeführten Patentdokumente angegeben.
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This Annex lists the patent family
members relating to the patent documents
cited in the above-mentioned inter-
national search report. The Office is
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of information.

La présente annexe indique les
membres de la famille de brevets
relatifs aux documents de brevets cités
dans le rapport de recherche inter-
national visée ci-dessus. Les renseigne-
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tif et n'engagent pas la responsabilité
de l'Office.

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP-A1- 414475	27-02-91	AU-A1-63449/90 WO-A1- 9102820	03-04-91 07-03-91
EP-A1- 388232	19-09-90	AU-A1-52783/90 CA-AA- 2012482 EP-TD- 388232 HU-AO- 902814 HU-A2- 54896 IL-AO- 93764 NO-A - 904712 NO-AO- 904712 PT-A - 93480 WO-A1- 9011089 FI-AO- 905591	22-10-90 17-09-90 02-05-91 28-03-91 29-04-91 23-12-90 30-10-90 30-10-90 28-09-90 04-10-90 12-11-90